University of Alberta

The effects of exposure of *Xenopus laevis* neural crest cells to low concentrations of ethanol *in vitro*

by
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To my father, the greatest teacher I know and my source of enlightenment
To my husband, Greg, whose love, laughter, encouragement and values will always guide and inspire me

Abstract

While numerous studies have focused on the effects of high concentrations of alcohol on neural crest cells, comparatively little is known about the effects of low concentrations of alcohol on this cell population.

Using various morphometric parameters, this study shows that neural crest cells, extracted from different cranio-caudal levels, are sensitive to alcohol (a concentration equivalent to a human blood alcohol level of one standard drink). However, cranial and trunk neural crest cells respond differently to this alcohol concentration. Although alcohol-treated cranial neural crest cells move away from the explant, the individual cell count is reduced. The displacement distance is also decreased and many morphological changes are observed. In contrast, trunk neural crest cells appear "less sensitive" under all of the growing conditions with alcohol only enhancing their migration on fibronectin.

Alcohol-induced morphological changes were quantified and representative photographs are presented. A comprehensive statistical analysis is offered in the Appendix.

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List of Abbreviations

ADH	aldehyde dehydroginase
BAC	Blood Alcohol Concentration
BMP	bone morphogenic protein
BSA	bovine serum albumin
N-CAM	neural cell-cell adhesion molecules
N-Caderins	neural Ca^{++} dependent cell-cell adhesion molecules
DNA	deoxyribonucleic acid
ECM	extra-cellular matrix
EGF	epidermal growth factor
EMT	epitheliomesenchymal transformation
ETOH	ethanol (alcohol)
FAE	Fetal Alcohol Effect
FAS	Fetal Alcohol Syndrome
FASD	Fetal Alcohol Spectrum Disorder
FGF	fibroblast growth factor
GD	gestational day
GM1	ganglioside
Integrins α β	cell-matrix adhesion molecules (α_x β_y - many types)
MDA	malondialdehyde
mg	milligram (10^{-3} gram)
ml	milliliter (10^{-3} liter)

mm	millimeter (10^{-3} meter)
mM	millimole (10^{-3} mol)
pH	potential of hydrogen ion (the negative of the logarithm of $[H_3O^+]$)
r	rhombomeres
RA	retinoic acid
RNA	ribonucleic acid
ROS	reactive oxygen species
SOD	superoxide dismutase
TGF	transforming growth factor
TNG-R	tumor necrosis factor
µm	micrometer (10^{-6} meters)

I

CHAPTER ONE

INTRODUCTION, STATEMENT OF THE PROBLEM and RESEARCH GOAL

Page

CHAPTER I: Introduction, Statement of the Problem and Research Goal

1.1 Introduction

The face is a mirror of human individuality, a reflection of human emotions, a center of important senses, and in many cases a foundation for social interaction. Its fundamental formation is the complex culmination of a nine-month gestation period.

The formation of the craniofacial region is complex and it may be influenced by internal and external factors[122]. When those factors are not favorable, craniofacial malformations may occur. The severity of those abnormalities can cover a broad range. In some cases, the malformations may be single and minor without functional significance, such as ear tags, microstomia or missing teeth but they may also be more complex, leading to significant disability or even death as seen in anencephaly[122].

Over the years, many causes of craniofacial birth defects have been identified. For example, a single abnormal gene can cause craniofacial malformations in syndromes such as DiGeorge Syndrome, and Treacher Collins Syndrome[61 62]. Abnormalities in the number or structure of chromosomes can also lead to compromised craniofacial development; for example, in the Trisomys 21 and - 13[62]. Craniofacial abnormalities may also result from damaging environmental factors which are known as teratogens. Examples are drugs, radiation, smoke and alcohol[62 124].

Fetal Alcohol Syndrome (FAS), first defined in the United States in the early 1970's[63] is one of the most common and most preventable birth defects. In addition to cognitive impairment, which appears to be most significant, the syndrome expresses unique craniofacial manifestations. The facial traits of FAS include overgrowth of the upper lip with diminished or absent philtrum, wide-spaced eyes, a flattened nose, and a small midface[63]. When these deficiencies are present to a lesser degree (and are therefore more difficult to identify) they are referred to as Fetal Alcohol Effect (FAE)[107]. Currently most experts refer to the disabling effects of ethanol in pregnancy as Fetal Alcohol Spectrum Disorder or FASD which includes an array of alcohol related birth defects such as FAS and FAE[9].

Although numerous surveys have been conducted in the past decade to establish the incidence of FAS, its true occurrence still remains unknown. According to government surveys, in Canada there are at least 350 children born with FAS each year[100]. In the United States, the surveys indicate there are at least 5,000 infants born with FAS each year; and another 50,000 are born with FAE[13].

Recent studies indicate that the rate of alcohol use among pregnant women has declined in recent years[42]. However, given that half of all pregnancies in the United States are unplanned[3], and that 53% of women of childbearing age in the United States drink after conception but before they know that they are pregnant[123], it is inevitable that unless all women of childbearing age totally abstain from drinking, there will always be

some risk of a fetus being exposed to alcohol, and therefore of a baby being born with FASD. For this reason, it is essential that the mechanism of alcohol's effects upon the developing fetus be determined so that clinicians might be guided towards new or improved treatments and more desirable health outcomes.

1.2 Statement of the problem

Although human development may be affected by maternal alcohol consumption throughout the entire gestation period, the typical craniofacial features of individuals with full FAS suggest that the craniofacial region may be especially sensitive to alcohol during the first three to eight weeks of development, when the formation of the head occurs[122]. Because neural crest cells are the main cell population in the craniofacial region during this period[31 49 65], they may be particularly susceptible to the effects of alcohol[19 67].

Neural crest cell damage following exposure to high concentrations of alcohol mimicking binge-drinking or chronic uptake has been well studied using different animal models[19 84 108 126]. In contrast, the effects of lower levels of alcohol consumption or less frequent use of alcohol have not been studied to the same extent and consequently are poorly understood. In the past, attempts have been made to study the consequences of low concentrations of alcohol on neural crest cells *in vivo*[21] but the results are inconclusive because it is difficult to measure the exact levels of alcohol reaching the fetus and the fetal exposure time to these alcohol levels has not yet been established.

There has also been little cell culture work completed because the cellular effects associated with an exposure to low concentrations of alcohol are difficult to study. In particular, there appear to be only three reports investigating neural crest cell exposure to low concentrations of alcohol *in vitro*[34 50 51]. Each of these studies concluded that low concentrations of alcohol mimicking social drinking cause significant morphological and functional changes in neural crest cells in culture. However, further study is needed for three major reasons.

First, the cell culture research that has revealed changes in neural crest cells after exposure to low or moderate concentrations of alcohol has only used one substrate as a support for cell growth. This is a limitation because during development the extracellular environment with which neural crest cells interact is more complex. For example, neural crest cells in the trunk migrate on a variety of extracellular matrix molecules such as fibronectin, collagen, and laminin[10 33 72 136 140] while neural crest cells in the head seem to be more selective. It has been shown that avian cranial neural crest cells migrate effectively both on fibronectin and laminin[72], while amphibian cranial neural crest cells migrate only on fibronectin[5]. Thus, the effects of low concentrations of alcohol should be tested with various substrates, as cell behavior seems to be substrate specific.

Secondly, two of the studies that have investigated low concentrations of alcohol tested the effects on neural crest cells in the presence of the neural tube[50 51] while the other study investigated neural crest cells 48 hr after the removal of the neural tube[34]. In

all of these studies, neural crest responses to alcohol may have been influenced by cues from the neural tube rather than the alcohol in the medium. In addition, testing neural crest responses 48h after their migration is missing the exposure period between neural crest pre-migration and early migration phases and therefore this study does not shed any light on the key steps in the early development of craniofacial structures.

Finally, two of the above studies did not use statistical analyses when evaluating the data and their results were solely based on descriptive analysis[50 51]. This limitation significantly undermines the validity and reliability of the investigations.

To overcome these restrictions, the effects of low concentrations of alcohol on neural crest cells should be tested using appropriate testing methods and statistical analysis. These changes could then provide stronger evidence that low alcohol levels may be detrimental to neural crest cells and especially normal craniofacial development.

1.3 Research goal

Using the animal model *Xenopus laevis*, this study evaluates neural crest cell growth characteristics in culture in the presence of an alcohol concentration of 0.05% (equivalent to one standard drink, for calculations see Appendix B) given at the time of explantation. Several different culture substrates are used. The study also allows for observation of a large number of cells which enables appropriate and valid statistical analysis to be completed.

Alcohol-inflicted changes to neural crest cells in humans seem to affect only neural crest cells in the craniofacial region leaving other areas of neural crest cells apparently unaffected as manifested by the symptoms associated with FASD[63]. Since trunk and cranial neural crest cells have different developmental fates[15 95 117] and behaviours[72] this may account for why they may respond differently to alcohol. Consequently, both trunk and cranial neural crest cells have been tested.

The specific research question is: What differences exist between *Xenopus laevis* cranial and trunk neural crest cells extracted from stage 19-21 embryos when exposed to an alcohol concentration of 0.05% in culture under different growing conditions? To answer this question this study evaluates: number of individually emigrated cells (1), cell shapes (2), displacement distances (3), and surface areas of individual cells (4).

The hypothesis is that if cranial and trunk neural crest cells are exposed to low concentrations of alcohol, then significant morphological changes will occur in both cell populations because both cranial and trunk neural crest cells are sensitive to low concentrations of alcohol. However the severity of those changes will depend on the neural crest cells anterior/posterior axial position because cranial and trunk neural crest cells are different cell populations.

II

CHAPTER TWO

BACKGROUND LITERATURE REVIEW

Page

CHAPTER II: Background Literature Review

2.1 Formation of neural crest and derivatives

Neural crest cells can be identified after gastrulation when the embryo is a trilaminar disc composed of ectoderm, mesoderm, and endoderm. Cells in the ectoderm form the neural plate around the dorsal midline of the embryo and its lateral margins rise up to form the neural folds which contain the neural crest cells[28 89 134].

At this stage of development the fate of neural crest cells has not yet been fully determined. For example, Selleck and Bronner-Fraser have shown that neural folds cells can also give rise to neural tube cells and epidermis, in addition to forming neural crest[115]. Similarly, cells of the neural plate can still give rise to both neural crest cells and neural tube derivatives at this stage[29].

Subsequently, neural crest cells migrate throughout the body and give rise to a wide variety of tissues. In the trunk, neural crest cells contribute mainly to Schwann cells, neurons and pigment cells[15 117]. In the head, the jaws and dentin of the teeth are derived from neural crest cells as are other derivatives, for example connective tissue and the muscles[31 49 65].

Although in higher vertebrates both the maxilla and mandible develop from neural crest cells, there is a distinct difference between the origins of the neural crest involved. The maxilla and Meckel's cartilage of the mandible are formed entirely by

neural crest cells derived from the posterior midbrain. The mandible itself is formed from neural crest cells derived from both the posterior midbrain and rostral hindbrain; the hyoid bones are formed from even more caudal neural crest cells[31 65].

The peripheral part of the sensory nervous system is also derived from neural crest cells [141]. Although some studies indicate that all sensory ganglia associated with the cephalic sensory system are exclusively of neural crest origin, other studies have demonstrated that some sensory ganglia, e.g. the trigeminal ganglion, are a combination of neural crest cells and cells from epidermal placodes[6 32].

The regional sources of the neural crest cells that contribute to the formation of the above mentioned cranial sensory nervous system have been mapped. For example, Lumsden et al[74] have shown that the neural crest cells contributing to the trigeminal ganglion originate from rostral levels. Neuronal neural crest cells from more caudal levels form the geniculate/vestibulo-acoustic ganglia, and neuronal neural crest cells from even more caudal levels form the superior and petrosal ganglia of the cranial nerve and the ganglion of the IXth nerve[74].

The parasympathetic ganglia of the craniofacial region also derive from cranial neural crest cells. Nerve cells originate from neural crest cells in the area of the mesencephalon and form the ciliary, otic, submandibular, lingual, ethnoid and sphenopalatine ganglia[85 93].

The capacity of neural crest cells to form various structures shows their highly pluripotent potential and their importance in developmental biology.

2.2 Front to back differentiation; what makes the head

The complex mechanism by which the craniofacial structures develop is not yet fully understood. There are many factors that contribute to this complexity:

- Genes (e.g. *Hox, Krox*-20, *Msx-2, Dlx, Shh* and others)
- Retinoids (vitamin A derivatives)
- Growth factors
- Plasticity-commitment

Genes

An array of genes is involved in craniofacial formation. *Hox* genes are expressed in the developing hindbrain and its corresponding pharyngeal region. While they are not expressed more rostrally in the developing craniofacial area[76 98 99] they are essential for the initial segmentation of the brain and neural crest cells converting rostral to more caudal areas. In the mouse for example, mutations of *Hox* genes have produced stunning malformations such as the complete omission of the skeletal elements derived from pharyngeal arch 2[44 105].

The *Krox*-20 gene with an expression domain in rhombomere (r) 3 and r5 in higher vertebrates and r5 in lower vertebrates may also play an important role in the early morphogenesis of craniofacial structures. Mutation of the Krox-20 gene in mice, for example, results in disruption of r3/5 development, leading to errors in morphological segmentation[113 130]. Furthermore, disruption of this gene results in abnormal expression of some of the *Hox* genes in r3 and 5. Therefore, abnormal *Krox*-20 expression may disrupt craniofacial formation through its direct effect on *Hox* genes.

Graham et al[46] have reported that *Msx-2* genes may also be important in early craniofacial development. During early stages of avian craniofacial development, it has been shown that apoptotic cell death in r3 and r5 creates a cell-free zone between the streams of neural crest cells leading to strictly individualized groups. They attributed the absence of neural crest cells in these areas to *Msx*-2 genes because these genes are strongly expressed at the onset of apoptosis in those regions *in vivo*. Furthermore, upregulating *Msx*-2 genes in explant cultures of r3 and r5 also results in apoptosis of neural crest cells[46].

Abnormal functioning of *Msx* genes has been shown to cause severe craniofacial malformations in various animal models. Null mutations of *Msx*-1 genes in mice cause malformations such as clefts, loss of palatine shelves, maxillary and mandibular hypoplasia and tooth malformations[112 139]. Overexpression of *Msx* genes in *Xenopus* has resulted in loss of the anterior structures including the eyes and sometimes the entire head[56].

Another group of genes that may be important in the formation of normal craniofacial structures is the *Dxl* gene group. For example, Qiu et al[102] found that mutations of these genes in mice resulted in an abnormal phenotype. The bones that normally derive from pharyngeal arches 1 and 2 were either deleted or altered. Other studies have also demonstrated that mouse embryos lacking the *Dxl* gene have ectopic skull components and are even missing dentition[101].

Sonic hedgehog gene (*Shh*) also has been implicated in craniofacial formation. Chiang et al found that a null mutation of the *Shh* gene in mice resulted in the absence or significant reduction of skeletal structures of the face[27]. *In vitro*, the addition of *Shh* protein to the migratory substrate of trunk neural tube explants inhibited neural crest cell spreading, while the removal of *Shh* restored their normal behavior[132]. This was attributed to decreased cell-substrate adhesion, mediated by integrins. Thus, these findings suggest that *Shh* plays an important role in the regulation of neural crest cell adhesion and migration.

Retinoids

Experimental evidence indicates that retinoic acid (RA) is also essential for normal development of the head. RA has been detected in all major vertebrate species where it is distributed in different embryonic fields. It has been found in *Xenopus*[26], mouse[57] and chick embryos[57 133].

In retinoid-depleted embryos the migration pattern of neural crest cells is significantly affected[77]. Although the pattern from anterior rhombomeres is normal, the groove between pharyngeal arches 2 and 3 is not formed and the cells from r5 to 7 are absent. There is also extensive cell death among neural crest cells along the whole neural axis, and the dorsal root ganglia fail to develop properly. These results strongly suggest that RA supports the survival and proliferation of neural crest derivatives, and that RA may also interact with the genes of apoptic pathways. It is noteworthy that deficiencies in RA do not affect the pharyngeal arch one, where *Hox* genes are not expressed (see above references).

Similar findings have been reported regarding the effects of high levels of RA on craniofacial development. In mice, excess levels of RA have been shown to affect *Hox* genes, resulting in dismorphogenesis of the hindbrain and of neural crest derivatives[82 83]. A more recent investigation is in agreement with these findings[30]. A single teratogenic dose of RA alters the expression of *Hox* genes in mouse embryos as early as 4 hr following the treatment. These results have been duplicated in birds[129]and *Xenopus* embryos[40 119].

Growth factors

During their migration, neural crest cells interact with a variety of tissues and factors. A number of secreted factors expressed by the surrounding tissues (e.g. fibroblast

growth factor [FGF], transforming growth factor [TGF], and epidermal growth factor [EGF] among many others) have been shown to play an important role in the formation of craniofacial structures. For example, mutation of the TGF genes in mice has been associated with cleft palate[111]. Bone morphogenetic protein (BMP), a protein associated with *Msx* transcription factors, has been shown to induce neurogenesis from neural crest cells[118]. BMP is also important during skeletogenesis. An excess of BMP has been shown to cause bifurcation of skeletal structures[8] and deficiencies of BMP have been reported to induce skull abnormalities[75].

Plasticity-commitment

Although many genes and various factors regulate embryonic craniofacial development, as described above, there is controversy over whether or not neural crest cells are pre-programmed. An early experiment by Noden[93] seems to demonstrate that the fate of neural crest cells is pre-programmed. This implies that the genetic information package is established prior to cell migration and is carried passively by the migrating neural crest cells. Support for this model has come from grafting experiments. For example, when Noden[93] transplanted the mesencephalic neural fold (which normally produces neural crest cells which colonize pharyngeal arch one and participate in the formation of the lower jaw) to the hindbrain (approximately the otic level of the rhombencephalon), the neural crest cells, despite their ectopic location, still generated

derivatives characteristic of their origin. In fact, a second set of lower jaws including Meckel's cartilage developed while the endogenous hyoid bone was reduced.

A more recent study by Couly et al[31] confirms Noden's findings. When Couly et al. transplanted more-rostral quail mesencephalic neural folds and associated dorsal neural tube to r4-6 of a stage-matched chick, he duplicated some of the lower jaw structures. In this experiment, the mesencephalic crest cells failed to express any of the *Hox* genes which would normally be expressed in the more caudal r4-6 region. Furthermore, when Couly et al[31] removed neural folds from the area of the presumptive mesencephalon plus the first three rhombomeres, neural crest cells from the more caudal r4-6 area colonized the rostral pharyngeal arch one area but still expressed their corresponding *Hox* genes suggesting that they also did not change their fate when moved to a more rostral location. However, in contrast, when Couly et al[31] transplanted the neural folds alone from the mesencephalon to the r4-6 level, the ectopic rods of quail cartilage that formed were disorganized suggesting that the neural tube may be critical for the patterning capacity of the neural crest to be fully expressed.

Noden's[93] experiment may have worked because the graft of neural fold contained neural tube tissue. In addition, when the most rostal part of the grafts (corresponding to the mesencephalon) was transplanted posteriorly in either of the experiments, there was no apparent maxillary process present in the new location. No explanation has been given for this finding[31 93].

Others suggest that neural crest cells may not be pre-programmed at all, but rather are plastic. For example, in birds, the rotation of r1-r7 changes neural crest gene expression, allowing for normal development of craniofacial structures[59]. However, the results are not always consistent because the rotation of r3-r7 results in abnormal gene expression for the new location which appears to contradict the above conclusion[59].

Trainor and Krumlauf[135]have provided important insights into the plasticity model of neural crest cell programming by studying neural crest cells from pharyngeal arch one (which do not express *Hox* genes) and two (which express *Hox* genes). A unique *in vivo* analysis showed that single or small groups of neural crest cells are plastic with respect to the *Hox* gene expression when transplanted between pharyngeal arches[135].

Whatever the mechanism by which a single neural crest cell is induced to alter its fate, neural crest cells in the head probably respond as a group rather than as single cells. If responses were dependant on each individual cell's level of differentiation, craniofacial embryonic development would be chaotic. If individual cells or even small pockets of cells act independently, expressing genes that are not being expressed in the surrounding cells at the same time, this would lead to a greater opportunity for errors, and thus local defects or even gross abnormalities in the craniofacial region.

Bronner-Fraser and Stern[16] have shown that the behavior of neural crest cells in the trunk is quite different from those in the cranial region. To establish the regulation of trunk neural crest, avian segmental plates or neural tube were rotated. The results

showed that the subsequent migration of neural crest cells in the trunk is dictated by their new locations. This finding is a great breakthrough in understanding neural crest cells as it may indicate that trunk neural crest has greater adaptability than cranial neural crest and may therefore be more resilient to environmental insults.

2.3 Patterns of migration

Neural crest cells are a dynamic cell population. As they emerge from the neural folds they begin to spread in an organized pattern along characteristic pathways until they reach their final destinations. The pattern in the chick[92], the mouse[96 116] and amphibians[12 109] have been well documented.

Although both trunk and cranial neural crest cells migrate in organized patterns, the migration of neural crest cells in the cranial region is different from that in the trunk. In the trunk, neural crest cell migration is affected by the somites, which results in neural crest segmental organization[104]. In the head, neural crest cells migrate in the absence of somites but still maintain organized groups. It is the formation of the forebrain (prosencephalon), the midbrain (mesencephalon) and the hindbrain (rhombencephalon) that plays a central role in cranial neural crest organization. The cells that contribute to the formation of the frontonasal area are derived from the mid- and posterior forebrain while the caudal midbrain and the hindbrain contribute to the formation of the pharyngeal arches[73]. The segmentation of the hindbrain into a series of

neighboring units called rhombomeres (r1-r8) is critical in the formation of the pharyngeal arches[65]. Initially, they form as a series of elongated fingers of tissue, each covered with ectoderm on the outside and endoderm on the inside with the exception of pharyngeal arch 1 which is covered with ectoderm on the inside. The core also consists of mesoderm to which neural crest cells are added when they leave their initial location[109].

Although the overall migration pattern of cranial neural crest cells is similar between various vertebrates, some significant differences do exist between species.

In birds, the first stream of cells (pharyngeal arch one), which contributes to the formation of the maxilla, the mandible and the trigeminal ganglion[31 65 74], is mainly composed of neural crest cells from r1, r2 and the posterior midbrain. The second stream of cells (pharyngeal arch two), which contributes to the formation of the hyoid bone, and geniculate/vestibulo-acoustic ganglia[31 65 74], is mainly composed of neural crest cells from r4. The third stream of cells (pharyngeal arch three), which also contributes to the development of the hyoid bone as well as to superior and posterior ganglia of the cranial nerve and ganglion of the IXth nerve, is composed of neural crest cells originating in r6 and r7[31 65 74]. Unlike those in other rhombomeres, in higher vertebrates, some experiments have shown that neural crest cells in r3 and r5 die resulting in the formation of a crest-free zone between the cells designated for different arches[47 48 74] (also see Section 2.2). However a series of labeling experiments demonstrated that r3 and r5 are not

completely devoid of the capability to produce migrating neural crest cells and those neural crest cells that do migrate contribute to the neural crest streams from adjacent even-numbered rhombomeres[11 90 110 114].

In *Xenopus*, as in birds, cranial neural crest cells also segregate into three groups adjacent to specific rhombomeres. In contrast to birds, however, no distinct cell-free zone between the groups destined for different pharyngeal arches is observed but there appears to be an ill-defined grove between the pharyngeal masses[79 109]. It is not clear what restricts the intermingling of migrating *Xenopus* neural crest cells and targets them to specific pharyngeal arches. Smith et al. suggest that in amphibians, EphA4 and EphB1 receptors are involved in restricting the mixing of the third and second pharyngeal arch neural crest, thus forming boundaries between pharyngeal arches[120]. Another difference between *Xenopus* and higher vertebrates is that, in *Xenopus,* r3 and r5-derived cells do not die but rather contribute to the formation of the first stream of cells (pharyngeal arch 1) or fill the entire third stream of cells (pharyngeal arch 3), respectively[106 120]. Finally, pharyngeal arch 1 does not contribute to the formation of the maxilla (at least not at a tadpole stage) and pharyngeal arch 2 contributes to the formation of the ceratohyal cartilage rather than the hyoid bone which only forms in higher vertebrates; pharyngeal arch 3 contributes to the cartilage of the gills[109].

The timing of neural crest cell emigration also varies between vertebrates. In mice, cranial neural crest cells migrate while the neural tube is still at the open

neural-fold stage[88]. However, in birds and amphibians, early cephalic neural crest migration coincides with neural tube closure[109 115].

2.4 Role of active cell movement / Extra-cellular matrix (ECM)

In higher vertebrates, when neural crest cells are in the neural folds, they are joined to one another by intracellular junctions which progressively disappear before the cells begin to migrate[86]. Using trunk levels, Newgreen and Gibbins also showed that at the onset of migration from the neural epithelium, the cells undergo epitheliomesenchymal transformation (EMT) and acquire mesenchymal properties[86]. Studies have shown that loss of cell-cell adhesion molecules such as N-CAM (neural cell-cell adhesion molecule) or N-caderins (neural calcium dependent cell-cell adhesion molecules) accompanies emigration of the neural crest cells in both cranial and trunk regions in avian embryos[2 17]. However, using an immunocytochemical staining technique, Milos et al. demonstrated that N-CAM and caderin cell adhesion families are present in the *Xenopus* pharyngeal arches [79]. Interestingly, this raises a question as to whether the cells in *Xenopus* pharyngeal arches have already undergone epitheliomesenchymal transformation as seen in the trunk region at the onset of migration[87].

It has been suggested by Noden[94 95] that in higher vertebrates, as the embryo develops, cranial neural crest cells are passively transported by the growth of the

surrounding tissues. Similar observations have been made in lower vertebrates. Based on morphological and immunocytochemical data, Milos et al[79] also argued that in amphibian cranial neural crest the cells spread passively down the sides of the head but through a relocation that is driven by cell division which "pushes out" the cells ventrally between the ectoderm and the mesoderm. However, others argue that the cells are not passive but rather move through active migration, as they frequently change direction and have the capacity to reroute their migratory pathways[69 70]. Furthermore the latter studies show that the cells at the pharyngeal arch periphery fan out through their environment, appearing to be moving in ways that are independent of the surrounding tissues, thus suggesting active migration.

Changes in the local environment, especially during the early stages of development when the embryo is undergoing rapid modification, may also play an important role in neural crest cell movement. The ECM may be of particular interest because it may be controlling neural crest cell relocation.

During the early stages of embryonic development, the cephalic region has been shown to contain a variety of ECM molecules, including fibronectin and laminin[68]. These ECM molecules appear to be important in cranial development because injections with antibodies against fibronectin and laminin, in the lateral aspects of the cranial neural tube, lead to defects in neural crest migration[14]. Taken together, available data are consistent with the proposal that neural crest cells may have to interact with a wide range of ECM molecules.

Neural crest cells in both the trunk and cephalic regions have been successfully maintained in long-term cultures on a variety of ECM molecules. Neural cells from the trunk of both higher and lower vertebrates attach to, and migrate effectively on, collagen, fibronectin, and laminin[10 33 72 136 140]. However, cephalic neural crest cells are more discriminating and differ between species. For example, in lower vertebrates such as amphibians, cranial neural crest cells attach and migrate effectively only on fibronectin[5] while in higher vertebrates such as birds, neural crest cells attach and migrate effectively on both fibronectin and laminin[72].

Experimental evidence has indicated that transmembrane proteins called integrins ($\alpha_x \beta_y$), are significant in cell attachment to and migration on ECM molecules[60]. Many integrins promote neural crest cell attachment and migration *in vitro* (Figure 2.4).

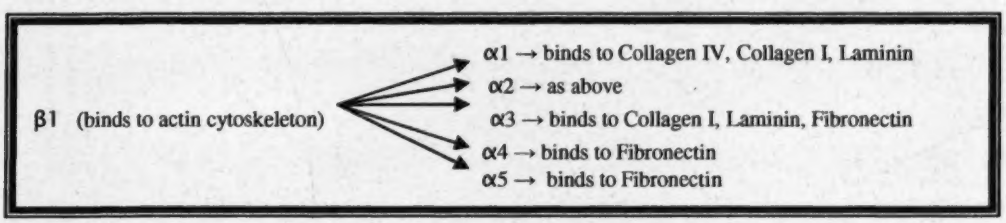

β1 (binds to actin cytoskeleton)
α1 → binds to Collagen IV, Collagen I, Laminin
α2 → as above
α3 → binds to Collagen I, Laminin, Fibronectin
α4 → binds to Fibronectin
α5 → binds to Fibronectin

Figure 2.4: Integrin mediated cell-matrix binding pattern (created by the author, based on discussion with colleagues)

The presence of both β and α subunits is necessary for neural crest attachment to take place. Some *in vitro* experiments have shown that avian neural crest cell attachment to fibronectin, laminin and collagen type I can be blocked effectively by the anti-β_1 integrin antibody[72], suggesting that this subunit is expressed on the cell membrane in both trunk and cranial regions. In contrast, neutralization of the α1 subunit blocks attachment of only trunk neural crest cells to collagen and laminin[72]. These data imply that trunk and cranial neural crest cells may not share the same mechanisms of attachment to extracellular matrices. However, there are some limitations to the idea that the α1 subunit may be important in cell migration and attachment because another study using mutations in murine embryos indicated that blocking the α1 integrin subunits has no effect on normal development[43]. Although species differences may offer a valid explanation of these phenomena, it is possible that the other α subunits may be more important in neural crest cell attachment and migration. Further research needs to be done to test the complex mechanisms involved in regulation of neural crest cell movement by integrins.

The steps involved in neural crest cell movement *in vivo* are still poorly understood. Much that is known about neural crest cell behavior derives from *in vitro* investigations of trunk neural crest cells[10 33 136]. In these studies, it appears that neural crest cell movement involves three distinct steps, similar to those seen in fibroblasts and fibroblast-like cells: first, the cell extends a leading protrusion, then it transfers the cytoplasm from the main body into the leading protrusion, and finally it detaches the trailing part.

Little research has been done on the connection between the efficiency of neural crest cell movement and the degree of attachment to the ECM. In the trunk, cell movements are severely restricted when the cells are highly adherent, or when adhesion is so weak that the cells are unable to generate enough traction for movement. Maximum migration speed and distance therefore occurs at an intermediate adhesion level[37 136].

2.5 Summary

Research to date seems to strongly support the following concepts:

- Trunk and cranial neural crest cells share some properties. Both are of neuroepithelial origin and require segmentation for organization and migration pattern.
- Different neural crest cell populations have some intrinsic differences, in particular developmental fates and their ability to interact with ECM molecules.
- In higher vertebrates, neural crest cell apoptosis is crucial for craniofacial development
- In the head there are numerous factors (e.g. genes, retinoids, growth factors, ECM) influencing neural crest cell migration and development.
- There is a controversy over whether or not neural crest cells in the head are pre-programmed.

2.6 Effects of alcohol on craniofacial development

In the course of the first three to six weeks of human gestation, normal craniofacial development is controlled by many internal factors (e.g. genes, growth factors, retinoids). However, when the embryo is exposed to environmental insults, the risk of abnormal development increases. The most obvious example of this is Fetal Alcohol Syndrome/ Effect (FAS/FAE): birth defects associated with maternal alcohol consumption during pregnancy. FAS is a collection of the most severe abnormalities[63], whereas in FAE the symptoms are present to a lesser degree. Currently the term Fetal Alcohol Spectrum Disorder (FASD) is used to cover the range of the outcomes associated with prenatal exposure to ethanol.

Some of the abnormalities associated with fetal exposure to alcohol are listed in Table 2.6.

Abnormalities	
Performance 1. Prenatal growth deficiency - length reduced proportionately more than for weight 2. Postnatal growth deficiency - lack of catch-up growth in spite of adequate nutrition 3. Developmental delay -social and motor performance related to mental, not chronological age	*Craniofacial dysmorphogenesis* 1. Microcephaly -with no significant catch-up through early childhood 2. Short palpebral fissures 3. Maxillary hypoplasia with relative prognatism 4. Cleft palate 5. Micrognathia 6. Narrow forehead 7. Small nose 8. Long upper lip with deficient philtrum
Other: 1. Cardiac anomalies 2. Joint anomalies	

Table 2.6: Abnormalities associated with fetal exposure to alcohol during early stages of pregnancy [63 128]

Although the main deficiencies of FASD are cognitive impairment and low birth weight, unique facial characteristics also develop in children exposed to ethanol during the early stages of development. The characteristic facial features of FASD include short palpebral fissures, an overdeveloped upper lip with thinned vermilion border and a diminished or absent philtrum. The maxilla also appears to be retrusive, giving the face a flattened appearance. The nose seems to be short with a low bridge and anterverted nostrils. The short upturned nose gives the impression that the distance between the nose and the upper lip is long[63 128].

The teratogenecity of alcohol on other craniofacial features (e.g. teeth, ears, muscles) has not been documented, suggesting that these malformations may be minimal or nonexistent. It has been suggested that alcohol may affect the craniofacial peripheral nervous system[19 20 38 39 67] but these conclusions have been drawn from animal studies and are yet to be confirmed in humans.

Taken as a whole, the craniofacial characteristics of children with FASD can be as distinctive as those in Down Syndrome. However, to appreciate the anomalies, they must be viewed as a unit rather than individually. The typical facial features of FASD are associated with high or chronic alcohol intake[78]; it is not clear how low levels of alcohol consumption affect the developing face and jaws.

2.7 Etiology of alcohol-induced craniofacial malformations

Anomalies associated with the characteristic facial abnormalities of FASD have been linked to alterations of certain embryonic cells during very defined periods of vulnerability (the embryonic stages of gastrulation or neurulation).

If embryos are exposed to alcohol during gastrulation, malformations are thought to occur as a result of neural plate deficiency[125 126 128]. In these studies, performed on mice, exposure of the embryo to alcohol reduced the size of the neural plate. Malformations of the forebrain occurred as well as short palpebral fissures, deficiencies of the philtrum and a long upper lip[125 126 128]. In the studies mentioned

above, Sulik and Johnson also showed that neural plate deficiency leads to an underdeveloped midface, possibly interfering with neural crest cell development at the edges of the neural plate. These anomalies mimicked FASD characteristics in humans[63].

However, when the exposure to alcohol occurs during neurulation, the craniofacial malformations are thought to occur because alcohol directly affects neural crest cells. Using various animal models, including the salamander, chick and mouse, several research groups have consistently shown that alcohol induces significant morphological and dynamic changes in neural crest cells[23 51 108 127]. Furthermore, alterations to neural crest cells can explain many of the craniofacial malformations associated with FAS.

Because the specific mechanism of action of alcohol on neural crest cells is not yet fully understood, a number of studies have investigated the effects of alcohol on various neural crest cellular responses and organelles. These range from cell death, to the effects on the cell membrane and cytoskeleton. Effects on various genes and signaling molecules have also been studied. In the following sections these will be discussed in turn. The literature has been reorganized according to subject so some papers are cited multiple times in different sections.

2.8 Cell death

Extensive research has been done on the effects of high alcohol concentrations on fetal craniofacial development. Much of this research has linked the characteristics of alcohol-induced craniofacial malformation to neural crest cell death. Cell death can occur by one of two recognized pathways: necrosis, a reaction to injury or disruption of cell metabolism which usually stimulates an immune response associated with inflammation; or apoptosis, self-programmed cell death. Cartwright and Smith[21], using detection methods for apoptosis (DNA end-labeling via TUNEL) and necrosis (Nigrosin dye), have demonstrated most interestingly that alcohol-induced neural crest death occurs by apoptosis. Needless to say the mechanisms regulating cell apoptosis are extremely complex and not well understood. Specific ligands and surface receptors (e.g. tumor necrosis factor [TNG-R]) or sensors residing in the cell nucleus and cytoplasm (e.g. cytochrome c) may be of particular importance because they seem to govern the activation of endogenous regulators of cell death such as caspases or a family of proteins represented by Bcl-2[52].

The studies that have tested neural crest cell viability when exposed to ethanol have used multiple experimental strategies, including cell cultures, whole-animal assays using transgenic mice, and *in ovo* chick experiments[19 21 35 38 39 67 124]. All of the study designs are important in testing the direct relationship between ethanol and neural crest cells, but *in vitro* methods have the advantage of being the most effective way of

analyzing the responses to ethanol at the cellular level. Conversely, since *in vitro* studies test only a small part of a very complex and integrated system, they may not give the whole picture (e.g. *in vivo*, *in ovo* or whole-animal assays). It is therefore important to use a combination of experimental designs to achieve more comprehensive, valid and reliable results.

2.8.1 *In vitro* conditions

Chen and Sulik[23] tested mouse neural crest cell viability using trypan blue with different ethanol concentrations over various periods of time. Their study found that alcohol levels of 50mM (~ 7 standard drinks) caused significant cell death. At ethanol levels of 100mM for 24h, cell viability decreased by approximately 60%. A second study[25], obtained comparable findings: at the same alcohol concentrations over the same time frame (100mM for 24h), cell viability decreased by approximately 40%. The second study also found that even six hours of exposure to this level of alcohol caused cell death of approximately 5%. A third study[22] using the same experimental methods had identical results. Following a 6h exposure to 100mM alcohol the viability of cultured mouse neural crest cells decreased by about 5% and 24h following exposure cell viability decreased by about 40%. However, since these studies only tested alcohol levels of 50mM or more, they do not shed any light on the effects of low ethanol levels.

Comparable cell culture results were obtained using chick neural crest. Davis et al[34] found that more than 90% of cultured neural crest cells died after twelve hours of exposure to alcohol levels of approximately 40mM. Using the same animal model a few years later, Rovasio and Battiato[108], using an *in situ* end-labeling method and strict morphological criteria, observed that, as in mice, many cells formed blebs on their surfaces (a typical characteristic of a dying cell) following six hours of exposure to 150mM ethanol in culture. These cell characteristics were observed in more than 20% of ethanol-treated cells.

Davis et al[34] also demonstrated, that in culture, alcohol levels as low as one standard drink (7mM/0.05%) can produce significant cell death. Approximately 60% of cultured chick neural crest cells died in the presence of an alcohol concentration of 7mM by 12h. This study tested the cells in very limited conditions (one type of substrate and one type of medium), and although representative photographs were available the study did not quantify changes in cell morphology and viability. However, the study is particularly noteworthy in that it demonstrates that low alcohol levels do not appear to diminish the teratogenic effects of alcohol on neural crest cells.

2.8.2 *In vivo* and *in ovo* conditions

Several research groups have set up *in vivo/in ovo* experiments where they have been able to produce craniofacial malformations. The animal models used were mouse[38] [39] [126], chick[19-21] and amphibian[84].

In mice, two intraperitoneal injections of 25% ethanol in saline administered in doses of 0.015ml/gm of maternal body weight [yielding a maternal blood alcohol level that peaked at approximately 30mM (~ 4.5 standard drinks) 30 minutes after the initial injection, and at about 40mM (~6 standard drinks) 30 minutes after the second injection] produced embryos with microcephaly, and malformations of the nose, lip and philtrum[126]. Although this study revealed an obvious size reduction of the neural plate, Koch and Sulik[67] later demonstrated that the rim of the anterior neural plate (the site of the presumptive neural crest) was also affected. The study also found that the cell death pattern in the anterior rim appeared to be pathologically consistent with the subsequent observed craniofacial malformations, including cleft lip, maxillary hypoplasia and other median facial deficiencies. These results have been also replicated by more current experiments. Dunty et al[38] demonstrated increased apoptosis in many areas of the early embryo, including the regions of the neural plate, neural folds/neural crest, the pharyngeal arches, and the mandibular and maxillary prominences after exposure to high alcohol levels. Later Dunty[39], using the same methods, also revealed that ethanol induces neural crest cell apoptosis in the hindbrain, leading to malformations in cranial nerve development.

In the chick, a single exposure to 250µl of 10% ethanol produces the highest incidence of craniofacial anomalies with no significant increase in mortality[19]. The abnormalities include microcephaly and foreshortening of the frontonasal region and

upper jaw, anomalies comparable to those seen in mice. Using acridine orange, Cartwright[19] showed that these malformations are the result of neural crest cell apoptosis. Increased cell death occurred within all rhombomeres as well as around the otic vesicle and in the cranial mesenchyme. Another study by Cartwright and Smith[20] obtained comparable results.

Cartwright and Smith[19] have estimated that 250µl of 10% ethanol is equivalent to about 7.5-9.1mM (levels equivalent to one standard drink). However, a subsequent study showed that this was not the peak level. The peak level was actually greater than 100mM, and was reached after 30min. The embryonic alcohol concentration then gradually decreased to 9-11mM after 2 to 3 hr, and remained fairly constant for approximately 30 hr thereafter[21]. Interestingly, using acridine orange, Cartwright et al[21] also demonstrated that when the embryos were exposed to 250µl of 10% ethanol during gastrulation, the cells did not actually die until after migration.

Cartwright et al[21] also showed that injection of even 100µl of 10% ethanol resulted in significant cell apoptosis. By calculation, it might be expected that this would result in peak alcohol concentrations equals to 40% of the levels obtained using 250µl of 10% ethanol, equivalent to approximately 40mM. Regardless, it seems clear that cell death is caused by exposure to high levels of alcohol.

The results from more recent investigations seem to support this hypothesis. When chick embryos *in ovo* were exposed to alcohol concentrations of 150mM for 48 h,

70% of the embryos became abnormal[108]. The abnormalities included asymmetry of neural crest distribution, presumptive neural crest in the lumen of the neural tube and cardiac anomalies. In addition, as in mice, shell–less cultured chick embryos treated with the same alcohol levels even for just six hours showed a significant increase in cell apoptosis in both presumptive neural crest (17.6%) and in the first migrating neural crest (25%).

In contrast, when shell-less cultured chick embryos were placed directly in lower concentrations of alcohol (7mM and 70Mm ethanol), even for 48h, no significant effect on the neural crest was observed[108]. Although similar effects were found *in ovo*, this study did not measure embryonic alcohol concentration at any point of the experiment and therefore conclusions cannot be made about the actual alcohol levels to which the embryos were exposed. Unfortunately, in this study Rovasio and Battiato did not test the effects of lower alcohol concentrations on cultured neural crest cells[108].

Exposure of *Xenopus laevis* embryos to alcohol levels between 1-2% (~140mM – 340mM) produces craniofacial malformations similar to those seen in FASD[84]. Embryonic alcohol levels following exposure were not measured. However, exposed embryos showed reduced body size, reduced length of the brain, and hypoplasticity of the anterior end of the body, especially around the mouth. Also, when the experimental embryos were examined at stage 16 using scanning electron microscopy, significant anomalies were observed in the neural fold areas from which neural crest cells develop.

The effects of exposure of *Xenopus laevis* neural crest cells to low concentrations of ethanol *in vitro*.

Although the entire neural fold appeared to be abnormal, the neural folds in the trunk region appeared somewhat less affected than in the cranial region. This study did not directly investigate the mechanism involved in the development of the craniofacial malformations.

In summary, analysis of all of the *in ovo* and *in vivo* experimental evidence strongly indicates that avian, mouse and amphibian neural crest cells are sensitive to high alcohol levels [corresponding to binge drinking behavior in humans]. Unmistakably, these alcohol levels are detrimental to embryonic development regardless of differences between species or how the experiments are conducted.

2.9 Critical window

The question of when the neural crest cells are most sensitive to ethanol exposure has been investigated in various animal models. By injecting chick embryos with a single dose of 250µl of 10% ethanol at various stages of embryonic development, Cartwright and Smith[20] found that neural crest cell apoptosis differs at various stages of development. Exposure of avian embryos at the onset of gastrulation and nerulation (developmental stages 4 to 6) to ethanol for approximately 9 days, produced the highest number of craniofacial malformations (approximately 60% in each experimental group). Treatment at these stages induced cell death within the midbrain and the rostral rhombomeric cell populations. Cell death was greatest in r3 and 5, where cell death is

normally highest. Cell death was reduced in the most caudal hindbrain populations. In contrast, in the same study, ethanol exposure at stages 8 to 12 for approximately 8 days produced significantly less craniofacial anomalies and these did not increase with an additional 24h exposure. Thus, this study concluded that the difference in the number of malformations is stage-dependent. Other animal experiments seem to support the Cartwright and Smith[20] conclusion that there is a critical window when exposure to alcohol is particularly harmful. Kotch and Sulik found that mouse craniofacial malformations resemble FAS in humans when exposure to alcohol (2.8g/kgx2) occurred as early as gestational day 8 (GD8)[67]. They found that even a shorter exposure (11h) during this stage of development resulted in excessive cell death in the rhombencephalic neural folds which contributes to the formation of the craniofacial structures. Using the same methods, Dunty and colleagues have provided further evidence supporting the stage specific vulnerability of embryonic cell populations to ethanol-induced apoptosis[38]. They administered (by intraperitoneal injection) a teratogenic dose of ethanol to pregnant mice at various stages of gestation. The results revealed that mouse embryos are susceptible to alcohol-related birth defects when exposure occurs any time between gastrulation and neurulation (GD7-11, respectively). Maximal neural crest cell death in rhombomeres and pharyngeal arch one is found at 8.5 days of gestation. These results certainly seem sufficiently similar to support the "critical window" hypothesis.

2.10 Oxidative stress

Experimental evidence has revealed that excessive neural crest cell death and subsequent malformations can be significantly reduced using antioxidants, suggesting a free radical mechanism for ethanol-induced teratogenesis[23 24 34 66] .When cultured mouse neural crest cells were exposed for 16h to 100mM ethanol in conjunction with the antioxidant *N*-acetylcysteine, cell viability increased by approximately 25% compared to the alcohol treated group[24]. Using the same animal model, experimental procedures and similar exposure time (12h) but different free radical scavengers, Chen and Sulik[23] achieved comparable results. Co-treatment with antioxidants such as superoxide dismutase (SOD), for example, increased cell viability by approximately 12%, co-treatment with catalase increased neural crest cell viability by approximately 10%, and co-treatment with L-tocopherol increased cell viability by about 17%. Interestingly, exposure of cultured avian neural crest cells to 50mM ethanol for 12h produced even more striking results [34]. Co-treatment with SOD increased avian neural crest cell viability by approximately 70%, while co-treatment with catalase increased cell viability by almost 30%.

To investigate the role of free radicals *in vivo*, Kotch et al[66] exposed mice embryos (GD8) for 6h to ethanol concentrations of 100mM in culture. Blue formazan staining of the ethanol-treated embryos indicated the presence of free radicals throughout the embryo, particularly in the cranial neural folds. Significantly less staining was noted

in embryos co-treated with SOD. All of these experiments support the conclusion that free radicals may be involved in ethanol-induced teratogenesis.

The mechanism by which ethanol can induce free radical formations is still controversial. However some of the possible steps are summarized in Figure 2.10.

1. CH_3-CH_2-0H + **OH•** → CH_3-C•H -0H + H_2O
2. CH_3-CH_2-0H + **CYP2E1** → Reactive oxygen species (ROS)
3. CH_3-CH_2-0H + **Mitochondria** → Decreased Glutathione Peroxidase activity
   → Reactive oxygen species (ROS)

Figure 2.10: Summary of the possible pathways involved in ethanol-induced oxidative stress mechanism (created by the author from numerous sources). OH· - hydroxyl radical, CYP2E1- an example of a cytochrome, Mitochondria – a site of cellular respiration. For explanation see text.

The ethanol described in the first pathway of the above table may cause oxidative stress through reaction with hydroxyl radical (OH•), resulting in abstraction of the hydrogen atom from -CH_2- to form H_2O, leaving behind an unpaired electron on the carbon atom which in turn causes damage of various cellular components[4 103]. The second pathway shows that ethanol may cause oxidative stress through the formation of reactive

oxygen species (ROS e.g. superoxide [O2•], hydrogen peroxide [H_2O_2] or hydroxyl radical). The exact mechanism of this pathway is still under investigation. However it has been shown that ethanol produces ROS by overstimulating certain cytochromes (e.g. CYP2E1)[64]. The third pathway involves mitochondrial damage leading to decreased intracellular antioxidant capacity e.g. glutathione peroxidase[7] or increased ROS[55].

Although free radicals have been known to cause adverse effects on a variety of cellular components including DNA in animal tissues, there is a large body of evidence suggesting that the primary site of action of ethanol-generated free radicals on neural crest cells is at the cell membrane level (see below). It seems logical that alterations at this basic level could produce cellular disorder leading to an extensive variety of ethanol-induced cellular responses.

2.10.1 Cell membrane

Davis et al[34] suggested that ethanol might cause alterations in neural crest cell membrane structure and function. They demonstrated changes in cell surface morphology, loss of microvilli, and formation of blebs after alcohol treatment. Using a gamma scintillation spectrometer, Davis et al. also found that neural crest cell preloaded with radiolabeled ^{51}Cr and exposed to 50mM ethanol for 12h generated ^{51}Cr levels in the medium six times as high as in cells exposed to both ethanol and superoxide dismutase (SOD). These changes to the membrane structure were attributed to ethanol-induced ROS.

Rovasio and Battiato[108] obtained similar results. Following ethanol exposure, neural crest cells exhibited altered cell shapes and surface morphologies and formed blebs. Blebs have been reported by other workers to be the result of membrane changes in response to redox reactions[34]. These are important findings because they have opened a complex scenario of molecular rearrangements of cell membrane components as a result of ethanol exposure.

This theory has been tested on cultured mouse neural crest cells. Using alcohol concentrations ranging from 50 to 200mM, Chen et al[25] observed significant changes in cell membrane lipid lateral mobility (a measure of membrane fluidity). This change was observed after exposure to an ethanol concentration of 100mM (~15 standard drinks). This alcohol concentration produced the most noticeable changes in membrane fluidity 18h after the exposure. Pre- or co-treatment of cultured neural crest cells with GM1 ganglioside (which are stabilizing molecules embedded in the cell membrane) at concentration levels of at least 40μM significantly prevented not only changes in membrane fluidity but also cell death (by almost 20% and 26% respectively). A subsequent study by Chen et al[22] obtained identical results. The same exposure time and alcohol concentrations caused an identical increase in membrane fluidity and cell mortality. This study also demonstrated that 24h exposure to alcohol levels ranging from 50 to 200mM significantly reduced the cell membrane GM1 content. These studies support the theory that ethanol disrupts membrane integrity, perhaps by altering the GM1 ganglioside component[22 25].

Ethanol-related alterations in neural crest cell membrane fluidity may also be linked to lipid peroxidation. There is an extensive body of evidence that lipid peroxidation can affect the arrangement of both the lipids[71] and proteins[18] leading to malfunctioning of the cell membrane[138]. Lipid peroxidation occurs when polyunsaturated lipids, i.e. lipids that contain more than two carbon-carbon double covalent bonds, deteriorate when exposed to oxidation processes. The following diagram (Figure 2.10.1) shows that an ROS (e.g. hydroxyl radical) can remove the hydrogen atom from a methylene (-CH2-) group in the cell membrane, leading to changes in membrane chemical composition, possibly leading to changes in cell membrane structure and fluidity.

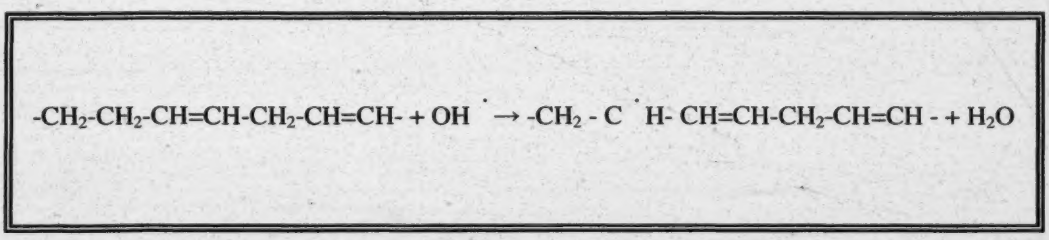


Figure 2.10.1: Representation of the initiation of peroxidation of a fatty acid with two double carbon-carbon bonds (crated by the author)

Kotch et al[66], using mice whole-embryo experiments, suggested that alcohol not only causes changes in cell membrane structure through lipid peroxidation but also that

these changes do occur in response to ROS. Using malondialdehyde (MDA) as an assay for measuring lipid peroxidation, Kotch demonstrated that 6h following exposure to 100mM ethanol, the uptake of MDA was almost 50% higher in ethanol treated embryos than in control embryos or in embryos co-treated with SOD. It was argued that alcohol generated free radicals can abstract hydrogen ions from lipids and once lipid peroxidation is initiated it can be a self-propagating process. Koch et al[66] also indicated that, although lipid peroxidation is usually confined to the site of production of free radicals, the low-molecular products of lipid peroxidation may diffuse in the plane of the membrane, spreading the lesion to distant sites and causing changes in membrane fluidity and permeability as well as changes in cellular function.

Another free radical that has been implicated in ethanol-induced neural crest cell lipid-peroxidation is the iron ion. However, the exact role that iron ions play in lipid peroxidation is, at present, an area of great confusion. Chen and Sulik[24] have demonstrated that exposure of neural crest cells to Fe (II) and Fe (III) had the same effect as exposing the cells to 100mM ethanol: in both cases neural crest cell viability decreased to approximately 50%. A combination of ethanol and Fe (II)/Fe (III) was even more damaging as it resulted in neural crest cell viability decreasing to 20%. The effects were significantly reduced in all three groups when the groups were co-treated with an antioxidant, increasing cell viability to almost 90%. Although this study did not directly test the effects of iron ions on neural crest cell membranes, the results strongly suggest

that iron ions can act like any other free radicals by causing lipid peroxidation in response to ethanol exposure.

The effects of ethanol-induced lipid peroxidation on neural crest cell mitochondrial membranes are also of interest because this intracellular organelle plays an important role not only in cell energy production but also in the initiation of apoptosis. Some studies have suggested that free radicals generated during ethanol metabolism alter mitochondrial membranes, leading to the release of apoptosis-promoting factors such as Cytochrome c[53]. However, the role of ethanol-induced formation of free radicals in the process of mitochondrial dysfunction and subsequent neural crest cell death is still contentious.

2.11 The cytoskeleton

Rovasio and Battiato[108], have shown that ethanol causes changes in neural crest cell shape, surface morphology, cytoskeleton and cell movement. These changes may be due to effects on the cytoskeleton. Cytoskeletal changes may in turn cause alterations to other morphological processes (e.g. attachment for various organelles, structural support, cell division).

When Hassler and Moran[50 51] cultured *Salamander* neural crest cells in the presence of alcohol as low as 7mM for 6 days they observed that the cells were not capable of undergoing normal morphological differentiation were although they were

capable of migration. The neural crest cell cytoskeleton appeared to be disorganized and both the tubulin and actin components of the ethanol-treated cells appeared to be affected. The microtubules were reported to be straighter and thicker, organized in parallel bundles and even absent from large areas of the cytoplasm. These changes occurred concurrently with an increased number of aggregates of pigment granules within the cells. The actin pattern also appeared to be disorganized. The normal linear actin filament arrangement was thickened within the cells, punctuated or absent in the proximity of the membrane, and irregular and clumped along the cell membrane.

Even more significant changes to actin filaments were observed in cultured avian neural crest cells. When Rovasio and Battiato[108] exposed avian neural crest cells for only 30min to an ethanol concentration of 150mM *in vitro*, the cells changed their actin filament arrangement. In 20% of the cells the microfilament bundles did not reach the cell periphery, compared to only 9% in the control group. Following 6h exposure, 22% of the ethanol-treated cells demonstrated a disorganized actin cytoskeleton. The actin filaments no longer followed a cross-linked arrangement. The number of actin filaments decreased, and the cells became shorter, blunter, and more condensed. Even more prominent changes were observed with longer ethanol exposure.

Similar effects on the neural crest cells cytoskeleton were also observed when Hassler and Moran[50] exposed neural crest cells to the drug cytochalasin which disrupts the actin cytoskeleton, causing the cell surface to detach from fibronectin[58]. This finding

suggests that ethanol-induced changes to the neural crest cell cytoskeleton may also interfere with neural crest cell attachment to ECM molecules. This in turn raises the question as to whether ethanol also alters the function of neural crest cell integrins. Because there is presently no research concerning the effects of ethanol on neural crest cell integrins, further research into this hypothesis is warranted.

2.12 Gene expression and other molecules

It has been demonstrated that ethanol causes neural crest cell death, producing craniofacial malformations associated with FASD (see Section 2.8). Cartwright et al[21] have demonstrated that chick neural crest cells die due to apoptosis. However the same study also showed that alcohol does not have any effect on the homeobox gene *Mxs-2* which in the chick is expressed only in the regions where apoptosis normally takes place (r3 and r5). This study also revealed that the levels of BMP-4 (a protein secreted from surrounding tissues which activates *Mxs-2*) did not increase following ethanol exposure; on the contrary, BMP-4 actually appeared to decrease. Therefore, this study concluded that ethanol-induced cell death may not involve the *Msx-2*/BMP-4 apoptosis pathway and that other apoptosis pathways may be involved. In the same experiment, Cartwright et al[21] examined the ability of apoptosis antagonists (in this case, capsase inhibitors) to prevent ethanol-induced cell death. They found that when caspase inhibitors were introduced into two cell samples - one a control group and the other exposed to ethanol -

the rate of neural crest cell death was very similar. In the control group 13 of 17 embryos (76%) did not demonstrate cranial neural crest apoptosis, while in the ethanol-exposed group 27 of 34 embryos (79%) showed no neural crest apoptosis. These results are counter-intuitive in that the ethanol-exposed group actually had a higher survival rate than the control group. Because of this, further experiments seem warranted.

Ethanol-induced neural crest cell death may also occur as a result of loss of signaling molecules expressed by the surrounding tissue such as those involved in neural crest cell adhesion. Ahlgren et al[1] demonstrated that the administration of high levels of ethanol to chick embryos resulted in craniofacial characteristics similar to those seen in embryos treated with antibodies that block *Shh* signaling. Both displayed reductions of the frontonasal process, hypoplastic pharyngeal arches and cranial neural crest cell death. Using in situ hybridization, Ahlgren et al[1] found that during the critical period of craniofacial development, ethanol has a significant impact on *Shh* signaling in the craniofacial region. The study demonstrated that after ethanol exposure, there was a significant decrease in *Shh* transcripts as well as a reduction some of the genes in the *Shh* signaling cascades. These changes were confirmed using reverse transcription (rt)-PCR comparison. *Shh* transcripts in embryos exposed to ethanol decreased by approximately 2-4 folds in the craniofacial region while Ptc transcripts decreased by 5-8 folds and Gli transcription factors by at least 6-fold.

Ahlgren et al[1] also found that exposing the chick embryos to ethanol had almost

identical effects to treating them with an *Shh*-blocking antibody. In both cases, the genes involved in *Shh* signaling were down-regulated. The only difference was that ethanol also reduced the Shh mRNA, which in turn appeared to cause a reduction in the production of *Shh*. The study concluded that ethanol results in a dramatic loss of *Shh* as well as the loss of transcripts involved in Shh signaling pathways.

The same study[1] also demonstrated that neural crest cell death was almost completely prevented when the ethanol treated cells were exposed to exogenous *Shh*: cell death decreased from 20% to less than 4% (a reduction of 80%). Furthermore, when ethanol treated embryos were treated with exogenous *Shh*, their frontonasal processes were almost identical to those seen in controls[1]. The control group was also treated with exogenous *Shh*, and cell death in this group dropped from approximately 4% to 2% (a reduction of 50%, but no error of measurement was given). Although a reduction of 50% could be viewed as significant, the study concluded that this reduction was not significant, and therefore that *Shh* might not be a limiting factor during normal development.

Nonetheless, this study did conclude that craniofacial abnormalities resulting from fetal alcohol exposure are caused at least partially by loss of *Shh* and subsequent neural crest cell death[1].

2.13 Retinoic acid

As previously discussed, alterations to retinoic acid (RA) levels can lead to fetal craniofacial malformations. Two studies looked at how ethanol exposure affects RA levels in the craniofacial region populated by neural crest cells[36] [127]. Both studies concluded that ethanol exposure affects RA levels. Sulik et al[127], however, concluded that the FAS craniofacial malformations are associated with increased RA synthesis, while Deltour at al[36] argued that the malformations are a result of decreased RA synthesis.

Sulik and others claimed that exposure of mouse embryos to alcohol produces craniofacial malformations that are somewhat similar to those of DiGeorge Syndrome (a condition associated with excess levels of RA)[127]. When Sulik et al[127] exposed the embryos to alcohol on their 7th day of gestation, they demonstrated FAS characteristics (long upper lip and no philtrum). When the embryos were exposed on day 8 1/2, the embryos' facial characteristics resembled those seen in DiGeorge Syndrome (short upper lip and the presence of a philtrum). Sulik et al[127] concluded that alcohol may over-stimulate RA production. However, our current knowledge of RA synthesis and ethanol metabolism indicates that ethanol actually inhibits (rather than stimulates) RA synthesis[36], invalidating Sulik's conclusion.

Deltour et al[36] exposed cultured mouse embryos to ethanol levels ranging from 10mM to 100mM. In the control group, 55% of the exposed embryos had some detectable RA. This number dropped to 41% in the 10mM group, and dropped even

further to 26% in the 100mM group. Deltour et al[36] stated that the drop from 55% (control) to 41% (10mM) was not statistically significant (no error of measuring was given), and therefore concluded that only the treatment with 100mM ethanol showed a significant decrease in RA detection.

Using whole-mount in situ hybridization, Deltour and others also observed that mRNA for aldehyde dehydrogenase (ADH), an enzyme used for both ethanol metabolism and RA synthesis, was present in the craniofacial region during normal development[36]. Therefore the study concluded that ethanol and RA may not only share the same biochemical pathways but also that both of these biochemical pathways may become competitive for ADH, resulting in a decrease in RA synthesis leading to craniofacial anomalies as seen in FAS. However, because these experiments were performed only on normally developing embryos, and not on ethanol-treated embryos, further research is warranted to support this concept.

2.14 Significant threshold

Although the maximal expression of full-scale FASD is clearly associated with high alcohol levels, the minimal level of ethanol needed to elicit those deleterious outcomes is still unknown. One study has suggested that in humans the minimal level might be quite low. Sood et al[121] concluded that many of the effects associated with FASD (aggression, delinquency, anxiety, depression and withdrawal) were observed even

after fetal exposure to levels as low as 1 standard drink per week[121]. These results indicate that low levels of ethanol may carry unknown risks. The significant threshold has yet to be adequately established.

The literature with respect to the effects of low alcohol concentrations on neural crest cells is sparse. Hassler & Moran[50 51] cultured salamander neural crest cells continually for 6 days in ethanol concentrations equivalent to one standard drink. Although the neural crest cells survived in alcohol levels of 0.05%, their ability to move, to assume a normal shape, and to maintain intracellular interactions (changes normally associated with high ethanol levels) was compromised at these low levels. These studies, however, have several flaws. The salamander neural crest cells were tested only on one substrate even though the ECM of the developing embryo is far more complex. The cells were also only cultured in saline so that even the controls appeared abnormal. And finally, the studies did not offer any quantification.

Davis et al[34] cultured chick cranial neural crest cells for 2 days and than subcultured them in the presence of an ethanol concentration equivalent to one drink. The cells' surface morphologies changed, and 60% of them died by 12 hr. However, similar to Hassler and Moran[50 51], the cells were tested only on one substrate and again the changes in morphology were not quantified .

Finally, Rovasio and Battiato[108] injected some early-developing chick eggs with an ethanol concentration equivalent to one standard drink, and exposed other shell-less

chick embryos to similar alcohol levels in culture. The changes observed were not statistically significant. Unfortunately Rovasio and Battiato[108] did not test the effects of low alcohol concentrations on neural crest cells in cultures.

2.15 Conclusion

Research on Fetal Alcohol Spectrum Disorder seems to strongly support the following concepts:

- Along with the effects of alcohol on a developing fetus such as performance deficiency and cardiac malformations, alcohol causes a wide range of effects on the development of the craniofacial structures derived from neural crest cells.
- Alcohol's mechanism of action is complex and multileveled. Some of the possible mechanisms are: free-radical induced cell apoptosis, alterations to the cytoskeleton, activation or blocking of the genes responsible for apoptic cell death/survival, and reduction of retinoic acid.
- Without doubt, chronic or heavy alcohol use has extremely hazardous effects on developing embryo and fetus.
- Low alcohol concentrations - levels that would be encountered during social drinking - are not well understood. These levels can still pose a significant risk to the development of neural crest cells, and therefore need to be investigated further.

- Until proven otherwise, professionals should assume that any fetal exposure to alcohol, especially during the first trimester, could have deleterious effects. Therefore, treatment efforts must carefully respect all fetal exposures to alcohol.

III

CHAPTER THREE

MATERIALS and METHODS

CHAPTER III: MATERIALS and METHODS

3.1 Animal model

The amphibian *Xenopus laevis* was used for this investigation for several reasons:

- As in higher vertebrates including humans, *Xenopus* craniofacial cartilage is derived primarily from cells of the cranial neural crest[109].
- Embryos undergo external development so they can be observed and manipulated relatively easily.
- The embryos are very large, aiding experimental treatment and observation.
- The embryos develop rapidly from fertilization through neurulation (~24h[91]), and the speed of their development can be regulated by changing the temperature. Therefore it is possible to obtain a desired stage of development, at a specific time.
- A large number of embryos is readily available at low cost.

The University of Alberta Health Sciences Animal Welfare Committee approved the use of animals for this study (Appendix D).

3.2 Animal housing

The Milos lab maintains an *X. laevis* colony which consists of 31 normal and healthy animals. These were purchased from NASCO (Fort Atkinson, WI in 1998). The animals are housed in pairs (male/female) in plastic mouse cages containing 12L of

water, in a partially shaded room with some natural daylight (Figure 3.2). The room temperature is maintained at 22°C. The cages are covered by plastic mesh secured with elastic bands.

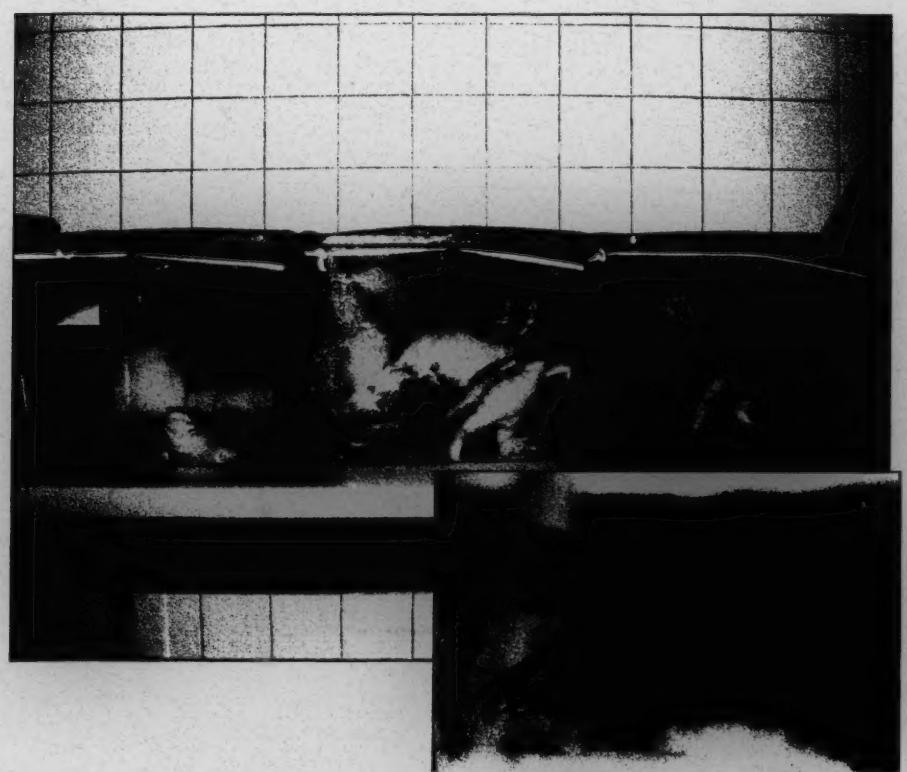


Figure 3.2: *X. laevis* from Milos lab colony. Insert: an adult pair of *X. laevis*.

Tap water is stored in 15 gal plastic pails for at least one day before use to release gases and to warm up to room temperature. 1 heaped tbs of Sodium Thiosulfate (Sigma) is added per pail and stirred in well before use to remove toxic agents such as chloride ions. Water in the animal tanks is changed twice a week to eliminate contamination.

Frogs are fed every other day with NASCO Frog Brittle. They are fed approximately 5-10 pellets each or until they are satisfied (as judged by the cessation of feeding). The pellets are scattered about or dropped directly above the animals to elicit a feeding response.

Breeding records are maintained for each pair. The *X. laevis* natural breeding cycle occurs between February and September when the male and female go into amplexus. This study took advantage of this and fertilized eggs were collected from spontaneously breeding pairs.

3.3 Embryo acquisition

In this study, fertilized eggs were harvested and placed in finger bowls containing the 24h-aged water. Abnormal eggs with irregular shapes, eggs with no separation between the animal and vegetal half, eggs with white spots, eggs with no cell division, and any eggs that had been attacked by fungus were removed with a wide mouthed pipette. Egg density was maintained at approximately 200 eggs per 100 ml of water. This allowed for appropriate oxygenation and aided in keeping contamination under control.

The eggs were incubated at 15°C for up to 72h, or kept on a bench top at 22°C for up to 24h. The embryos were then observed, and based on their identifiable external features, assigned a developmental stage according to Nieuwkoop and Faber[91]. Embryos at stages 19-21 were selected. However it should be understood that although staging based on the appearance of the embryo is strongly correlated with internal events, the correlation is not exact and therefore the stage assignments were more generalized.

All surgical procedures were performed on a normal bench top (Figure 3.3). The work area and all instruments were cleaned and disinfected with 70% ethanol before each use.

Figure 3.3: Working area and instruments for operating on embryos.

In preparation for dissection, the jelly layer of each embryo was manually removed using two pairs of fine, watchmaker's forceps. This manipulation was performed under a low-power binocular dissecting microscope as follows. The jelly was pierced with one prong of the first pair of forceps which were then closed and held firmly, thus grasping the jelly layer. Then, one point of the second forceps was inserted into the jelly alongside the first forceps and then closed. The jelly was then ruptured by pulling the two pairs of forceps apart. The vitelline membrane and embryo then emerged. The vitelline membrane was next removed by a similar method. The exposed embryos were then washed in two changes of Danilchik's medium (pH 7.4) containing the antibiotic gentamicin sulfate (50mg/L) (for medium preparation see section Appendix A).

Two-percent agar plates were made approximately one week before the experimental procedures (Appendix A). Agar was brought to a boil in distilled water and poured into watchglasses to a depth of 5mm. This agar layer was sufficiently thick to allow for the preparation of depressions to hold the embryo and so that the dissecting medium could cover the embryo but not overflow the dish. To prevent water evaporation and bacterial and fungal growth, the dishes were then covered with foil and refrigerated until needed.

3.4 Neural crest dissection

X. laevis embryos are difficult to dissect because their ectoderm can be very adhesive making it difficult to remove cleanly from the underlying mesoderm. This is important because the underlying mesoderm is extremely delicate and thus very vulnerable to damage during handling.

Patience and steady hands are needed to dissect cranial neural crest successfully from the embryos. In the past, tungsten microneedles were used in this laboratory to extirpate neural crest cells from amphibian embryos. However, it was found that cactus spikes of various diameters were more useful in extracting neural crest cell explants. Cactus spikes offer the sharpness, rigidity and the size required for easy manipulation and they also have the advantage of being less adhesive than tungsten microneedles, allowing for cleaner surgical procedures.

Cactus spikes were removed from the cactus plant (Echinopsis and Mammillaria species) using scissors and then attached to a wooden holder (long enough to grasp comfortably) using nail polish (Figure 3.4.1). Because cactus needles are brittle and lose their rigidity when held in the dissection medium for a prolonged period, a supply of cactus spikes was kept available in case the needles were damaged during operations and more were needed.

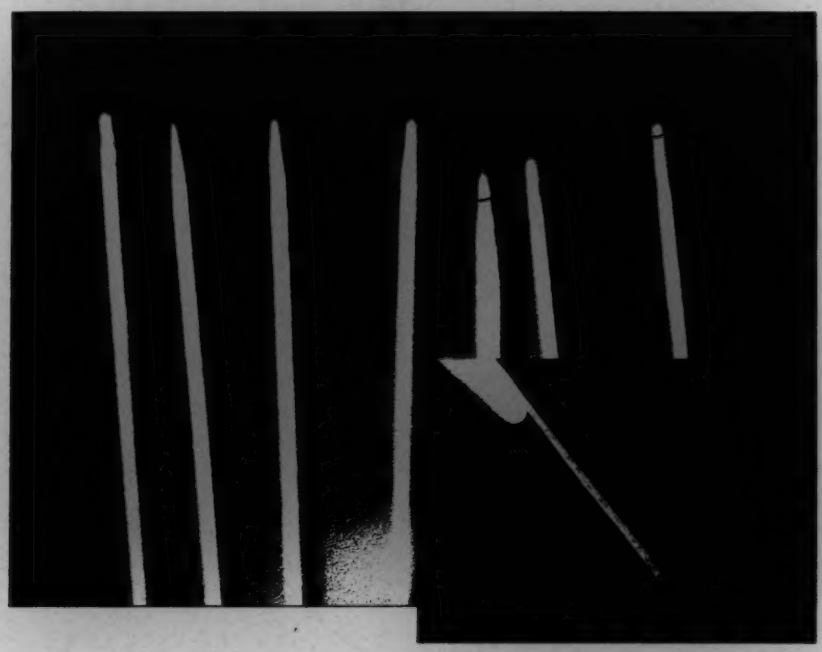


Figure 3.4.1: Dissection needles. Dissection needles (identified by the circles) were prepared from cactus spikes of various diameters. Insert: Dissection needle magnified with a dissecting microscope.

For culturing neural crest cells, embryos were transferred with a pipette into a shallow depression cut into the agar of operating dishes. The embryos were immersed in Danilchik's medium with antibiotics. To hold the embryo stationary during the operation and to orient the embryo in the best position for a precise dissection, the depression was of such a size that the embryo fitted into it snugly. To expose the neural crest and the surrounding tissues, the head and trunk ectoderm were stripped from embryos with the

cactus microneedles under a low-power dissecting microscope.

Neural crest was identified on the basis of its location and color as described previously[41 91 109]. The first distinguishing feature of *Xenopus* cranial neural crest is that it forms bulging cell masses lateral to the neural tube at stage 16[109]. There are three main segments corresponding to the mandibular arch (pharyngeal arch 1, which at stage 22 separates into two streams[91]), the hyoid arch (pharyngeal arch 2) and the pharyngeal arch material (which at stage 23 subdivides into pharyngeal arches 3 and 4[109]).

At stage 19 the bulging cell masses have enlarged and become clearly distinguishable from the surrounding tissues under the dissecting microscope. These lateral masses continue to grow and spread beneath the ectoderm and ventrally over the mesoderm in close proximity to each other throughout stage 21 (personal observation, [79 109]). At stage 21, the anterior border of the mandibular arch still lies dorsal to the posterior portion of the eye (personal observation, [91]).

A second distinguishing feature of cranial neural crest cells which is visible after ectoderm removal is their pale gray color. The pigment is derived from the mother and deposited in the egg during development, coming to lie in the ectoderm[91].

Trunk neural crest cells are premigratory at the above stages. Premigratory trunk neural crest is visible as a pale, gray strand located dorsal to the neural tube after the ectoderm is removed (personal observation, [91]). Lateral migration begins at stage 25 and the trunk neural crest gradually becomes depleted with further development[91].

For dissection of cranial neural crest, embryos were first positioned with their dorsal side up. After the cranial neural crest boundaries were identified, the embryos were slowly rotated with the right side up. (As a general rule the operations were performed on the right side only; the left side was used as a control to compare visually if all of the bulging masses of cranial neural crest cells were removed from the operated side). For dissection of the trunk neural crest, the embryos were positioned with their dorsal side facing up. Figures 3.4.2.a, b and 3.4.3.a, b illustrate the two areas of the embryo used for dissection.

1. Dissection of cranial neural crest (Figure 3.4.2.a, b):

- Cranial ectoderm was pierced posterior to the bulging cranial neural crest cell masses with the tip of a cactus needle. The needle was inserted gently and moved forward just underneath the ectoderm. The tip up the needle was gradually raised, thus stripping off the ectoderm and exposing the cranial neural crest (Figure 3.4.2.a). During this procedure the embryo was held in place with a second cactus needle. The removal of the ectoderm occurred readily as long as the underlying tissue was not punctured. If the cells from the underlying tissue were found on the stripped cranial ectoderm, the explants were excluded from the experiment.
- The bulging, pale gray cell masses were identified visually (Figure 3.4.2.a, yellow region). Holding the embryo down with one cactus needle, a single cut was made with another needle along the posterior border of the most distant neural crest segment (Figure 3.4.2.b, black dotted line), making certain that the mesoderm was not punctured. The explants were then teased from the surrounding tissue until the caudal boundary of the eye was reached. In this way all three neural crest segments were completely removed (Figure 3.4.2.a, red dotted line).

DISSECTION OF CRANIAL NEURAL CREST

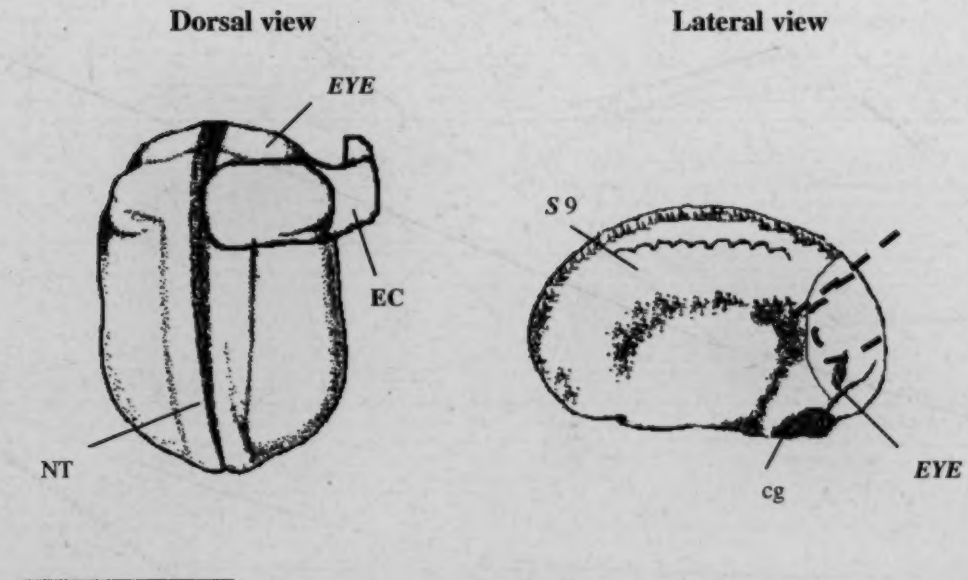
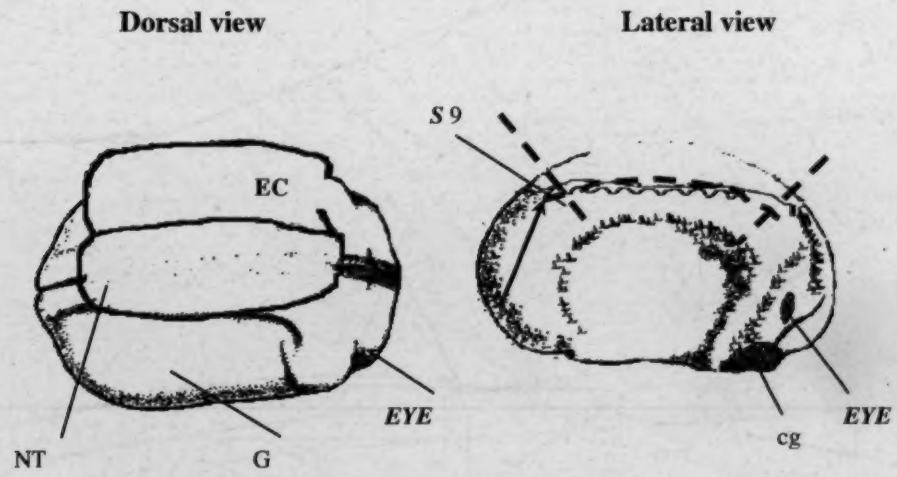


Figure 3.4.2: Dissection of cranial neural crest from *Xenopus laevis* embryo at developmental stage 21 (modified from Nieuwkoop and Faber, 1967). a) Dorsal view following the removal of ectoderm. The region of cranial neural crest is indicated by the yellow area. b) Lateral view shows isolation of cranial neural crest (yellow) along the doted lines (see text). EC = ectoderm; NT = neural tube which is completely closed at stage 21; S9 = position of the 9th somites; cg = cement gland. Bar: 1mm. See text for identification of cranial neural crest cells.

2. Dissection of trunk neural crest (Figure 3.4.3.a, b):

- The dorsal ectoderm was stripped off, exposing trunk neural crest (Figure 3.4.3.a). Holding the embryo down with one cactus needle, the ectoderm on the right side was pierced with a second cactus needle. The incision was made ventral to the trunk neural crest region, just above the position of the 6-9th somites (Figure 3.4.3.b, red arrow). The second needle was then gently pushed forward underneath the epidermis alongside the entire length of the trunk neural crest region. The epidermis was then stroked with the first needle alongside the second needle, splitting the epidermis. The epidermis was then peeled off toward the opposite side of the embryo just enough to expose the entire trunk neural crest. This process occurred easily as long as the mesoderm and somites were not punctured.
- Trunk neural crest was visually identified on the dorsal aspect of the embryo (Figure 3.4.3.b, blue region). Two transverse incisions were made (Figure 3.4.3.b, black dotted line), one at the hindbrain and the other at the most caudal pair of somites formed at the tailbud region (somites 6-9). Holding the embryo down with one cactus needle, the gray strand of cells was then cut with a second cactus needle along its lateral edges (Figure 3.4.3.b, red dotted line), teasing it away from the underlying tissue. As the dissected tissue curled up anteriorly it was removed from its location.

DISSECTION OF TRUNK NEURAL CREST



a) b)

Figure 3.4.3: Dissection of trunk neural crest from *Xenopus laevis* embryo at developmental stage 21 (modified from Nieuwkoop and Faber, 1967). a) Dorsal view following the removal of ectoderm. The region of trunk neural crest is indicated by the blue color. b) Lateral view shows isolation trunk neural crest (blue) along the dotted lines (see text). EC = ectoderm; G = gut; NT = neural tube which is completely closed at stage 21; S9 = position of the 9th somites; cg = cement gland; red arrow = initial incision between the lower end of the trunk neural crest region and just above S9. Bar: 1mm. See text for identification of trunk neural crest cells.

3.5 Culture conditions – Pilot study

A pilot study was conducted to establish the growing conditions and timing of development for cranial neural crest explants without alcohol. This was required because, although trunk neural crest has been grown successfully in this laboratory for a number of years in complex media[80 81 140], attempts to grow it in saline had resulted in explant attachment but no migration[140]. Cranial neural crest had also not been successfully grown (Milos, personal communication).

A recent study by Alfandari et al[5] reported the culturing of *X. laevis* cranial neural crest cells in Danilchik's medium containing bovine serum albumin (BSA) at pH 8.3, using fibronectin as a substrate. [This pH mimics the blastocoel pH of early *X. laevis* embryos [stages 6.5-7][137]. Originally Alfandari et al[5] used this pH to culture isolated *X. laevis* blastula fragments (Alfandari 2004, personal communication). Gillespie, 1983[45] has also indicated that the blastocoel pH of older *Xenopus* embryos [stages 19-21] measures 8.3, although there are no pH measurements available for the extracellular fluid of the *Xenopus* head.] A pH above 8 for the craniofacial region is considerably higher than physiological pH. Therefore, it was decided to repeat the experiment of Alfandari et al[5] but with the pH modified to 7.4, since it was felt that a lower pH is more representative of normal physiological conditions. The addition of BSA was also omitted because it was felt that inhibitors of nonspecific adhesion were not a necessary component. Collagen and fibronectin were used as substrates in this pilot study even

though Alfandari et al[5] had shown that at pH 8.3, cranial neural crest cells would not attach to collagen. This pilot study tested whether or not the cells would attach to collagen at pH 7.4. This study also tested this medium with trunk neural crest since attempts to culture it in simple saline had also not been successful[140].

3.5.1 Substrates for Pilot study

Petri dishes (35x10mm, FALCON 1008) were coated with Type I collagen from either rat-tail or calf-skin as follows (also see ref. [140]). Collagen (0.5mg/ml) was dissolved in 0.05% acetic acid in deionized water and added to each dish at a concentration of 0.25mg/dish (0.26μg/mm^2). After evaporation the dishes were sterilized by irradiating them for 48h with UV light. Then, before use, the dishes were washed three times with sterile, deionized water to decrease the substrate acidity.

Fibronectin substrates were prepared by dissolving 0.1% bovine plasma fibronectin (SIGMA Co.) in Niu-Twitty saline (NTS) at pH 7.4 (recipe for NTS in Appendix A). Fibronectin-coated dishes were also UV-irradiated and washed three times with sterile deionized water immediately before use.

3.5.2 Medium for Pilot study

Danilchik's medium was used at pH 7.4 (Appendix A). To maintain identical growing conditions two milliliters of this medium was added to each coated tissue culture

dish under sterile conditions. Explants obtained as in Section 3.5 were immediately transferred from the operating dish to the bottom of the culture dish using a glass pipette. It was important to place the explant beneath the surface of the fluid, because explants disintegrate if they touch the surface and are exposed to air.

3.6 Culture conditions - Main experiment

The pilot study demonstrated that cranial neural crest cells attach to and grow on fibronectin but not on collagen, at pH 7.4 (see Results). Consequently, Danilchik's medium at pH 7.4, with fibronectin as a substrate was used for the cranial neural crest experiment.

For purposes of comparison, trunk neural crest cells were also cultured on fibronectin in Danilchik's medium at pH 7.4. Because previous investigations have shown that trunk neural crest cells grow on collagen[140], this substrate was also used in Danilchik's medium. Finally, trunk neural crest explants were tested on collagen in an enriched medium consisting of L-15 Leibovitz medium supplemented with 20% fetal calf serum (SIGMA Co.) at pH 7.4. These latter conditions have been used previously[140] and this study was interested to see what the effects of alcohol would be on trunk neural crest when grown under these conditions.

Cultures were maintained at room temperature in a humidified air environment to prevent evaporation of the medium. To provide humidity, the petri dishes were placed in

oversized 100x20mm petri dishes containing two drops of distilled water, and covered with a lid. Cranial neural crest was cultured for 5h because the pilot study demonstrated that during this time the explants adhered to the culture substrate and the cells showed maximal outward migration, detaching thereafter. Trunk neural crest cells were cultured for 48h.

Experimental cultures were grown in these two media but with 0.05% ethanol (SIGMA Co.) added starting at T=0. This alcohol concentration was found to be experimentally effective in previous studies [34 50 51] and corresponds to an alcohol level equivalent to one standard drink (Appendix B).

Table 3.6 outlines the media, the substrates, and the number of explants used for cranial and trunk neural crest experiments.

Table 3.6: Procedure Flow Chart. Each dish contains cultures from an individual batch of embryos. Every dish contains 5 explants. N/A indicates that the cells do not grow under these conditions. The cultures are observed at set intervals. ETOH=ethanol (alcohol)

Medium	**Substratum**	**Trunk neural crest cells**		**Cranial neural crest cells**	
		24h	**48h**	**3h**	**5h**
Danilchik's Medium *Control* *or* *0.05 % ETOH*	Fibronectin	3 dishes 15 explants	3 dishes 15 explants	3 dishes 15 explants	3 dishes 15 explants
	Collagen	3 dishes 15 explants	3 dishes 15 explants	N/A	N/A
Enriched Medium *Control* *or* *0.05% ETOH*	Collagen	3 dishes 15 explants	3 dishes 15 explants	N/A	N/A

3.7 Observation of cultures

Because the explants were immersed in a fluid, the living cultures were observed with a Zeiss Inverted tissue culture microscope using phase contrast optics.

3.8 Photography

Cultures were photographed using the inverted microscope equipped with a Nikon digital camera (Figure 3.8). The magnification varied with the size of the culture so that the frame was filled with the area of interest. Each entire culture was photographed using a 25X microscopic magnification with a camera lens ranging from f/3.5mm to f5.1mm, and the individual cells were photographed using a 100X microscopic magnification with a camera lens of f/3.4mm, f/3.5mm or f/3.6mm to obtain best results. A standard grid was photographed with each magnification and used for calibration. The 25X microscopic magnification, with the camera lens ranging from f/3.5mm to f/5.1mm, was calibrated to ranges between 0.87 and 0.44μm per pixel. The 100X microscopic magnification, with a camera lens of f/3.4mm, f/3.5mm, and f/3.6mm, was calibrated to 0.25, 0.23 and 0.20μm per pixel respectively.

Color images of cranial neural crest cells were taken at 3 and 5h while images of trunk neural crest cells were taken at 24 and 48h. The two different measurement times for cranial and trunk neural crest cells were selected based on similar culture characteristics at those times. Both the entire cultures and individual cells were photographed at these times.

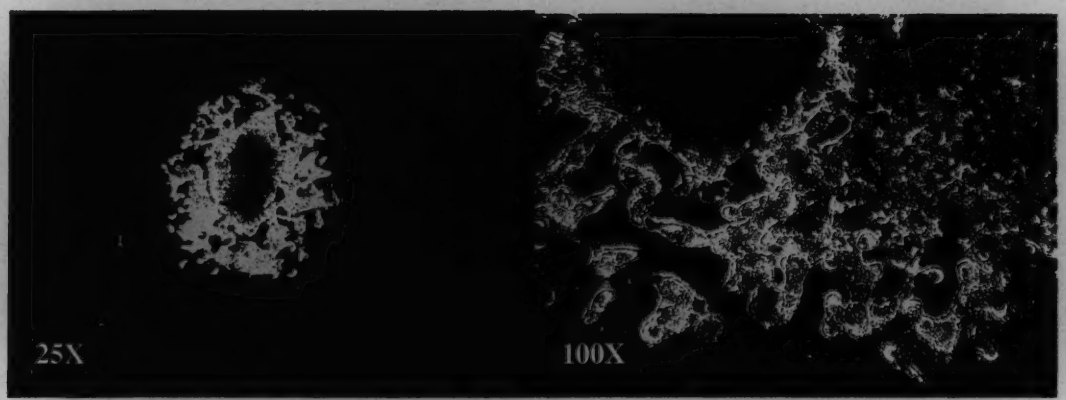


Figure 3.8: Typical image of the entire culture and individual cells.

3.9 Image analysis

A MetaMorph Office Imaging System (Universal Imaging Corporation) was used for image processing and analysis. Although MetaMorph supports both color images and images of unstained cells, some of the original images acquired with the Nikon digital camera required the use of MetaMorph's processing tools to make subsequent analysis more informative. Tools used to enhance the images included blue, red and green filters to improve color contrast and image sharpness.

The following data were obtained for each culture.

➢ Entire culture analysis

(1) individual cell count (excluding those in dense, multilayered epithelial sheets)

(2) cell shape:

- irregular cells with numerous cytoplasmic projections extending in various directions (Figure 3.9.1.a, b)
- elongated cells with leading sheets of cytoplasmic projection (Figure 3.9.1.c, d)
- round cells without cytoplasmic projections (Figure 3.9.1.e, f)

(3) extent of cell migration based on the 20 most displaced cells around each explant (furthest from the center of the explant, including cells at the peripheries of the epithelial sheet)

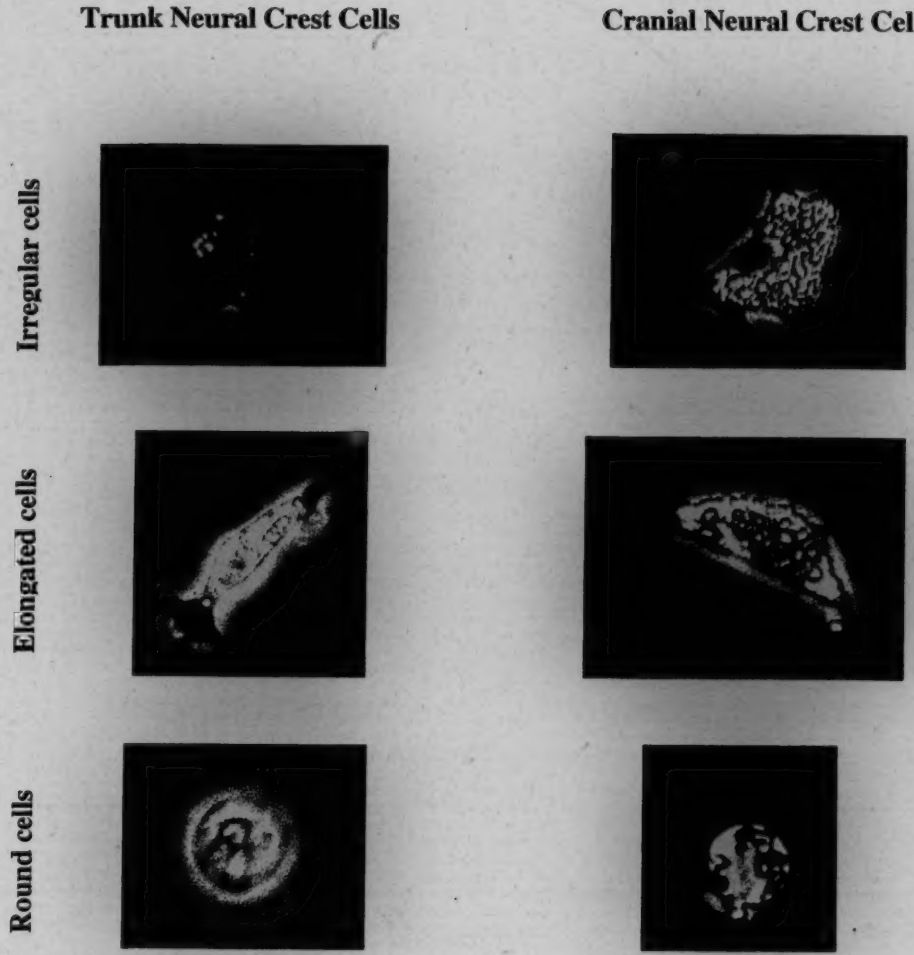


Figure 3.9.1: Cell shapes. a-b) Irregular cell shape with projections extending in multiple directions. c-d) Elongated cells with a leading sheet of cytoplasm extending in one direction. e-f) Round cells with no projections.

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- Individual cell analysis:

  (4) total cell surface area (spread), including filopodia (only cells with entire, well-defined peripheries were selected for this analysis).

Because the computer function "automated object measurements" (which utilizes an intensity threshold to limit and identify subject matter), could not be applied in the analysis of the color images, manual measurements were made using " regional measurement tools". The total number of cells, and the total number of cells in each shape category, were enumerated using the sub-function "manual object count". The total area (in square μm) of each cell was determined by tracing the cell border using the computer mouse and the freehand selection tool. Displacement distance (in μm) was measured with the single line tool by drawing a straight line between the two selected points (a cell found furthest from the explant and the explant center). The regional tools used for the total area and displacement distance measurements are indicated by the red arrows in Figure 3.9.2.

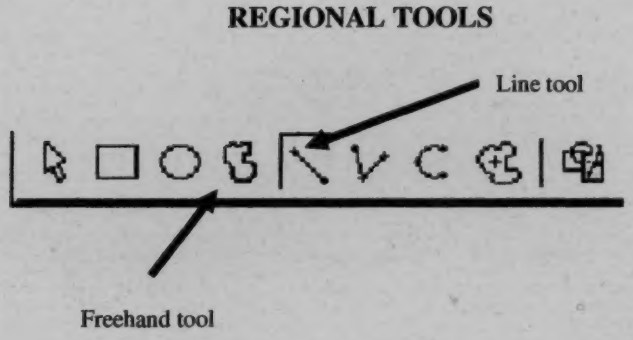

Figure 3.9.2: Regional tools used in methamorphic analysis.

3.10 Measurement error

Measurement error was addressed through intraobserver reproducibility. Two photographs were randomly selected from controls for this test: one image of an entire culture and one image of individual cells. Each image was evaluated by a single person (Joanna Czarnobaj) on six different occasions. Each evaluation was performed under the same conditions.

The image of the entire culture was examined for:

1. number of individually emigrated cells (excluding these in dense, multilayered cell sheets)

2. cell shape (grouped into three categories: irregular with centrally positioned nucleus, elongated with a leading cytoplasmic projection, and round)
3. extent of migration (average distance of the 20 most displaced cells around the entire explant [including these in multilayered cell sheets] from the center of the explant). The center of the explant was chosen following visual inspection. The measurement of error was not performed directly on this parameter. However, the accuracy of hitting the target was based on the measurement error for cell displacement (extent of migration) from the target point.

The images of individual cells were examined for:

4. cell surface area (only cells with entire, well-defined peripheries were selected)

Each measurement error was calculated based on mean values (see Chapter IV, Section 4.1).

3.11 Data analysis

The total cell count and the cell count in each shape category were calculated for 15 explants for all groups and compared statistically. Values were obtained from three independent experiments for each condition.

The extent of neural crest cell migration was estimated by measuring the linear distance between the center of the explant and the 20 most displaced neural crest cells, including those in the epithelial sheets. The values for each of the 15 explants were than averaged and statistically compared. Values were obtained from the three independent experiments for each condition.

For cell surface area, average values were obtained from 15 cultures for each set of conditions from the three independent experiments. The cell surface analysis was performed based on the total number of individually emigrated cells as shown in Table 3.11.

	Control Group	ETOH Group
Cranial neural crest cells 3h Fibronectin/Danilchik's Medium	271	37
Cranial neural crest cells 5h Fibronectin/Danilchik's Medium	444	202
Trunk neural crest cells 24h Fibronectin/Danilchik's Medium	358	359
Trunk neural crest cells 48h Fibronectin/Danilchik's Medium	298	321
Trunk neural crest cells 24h Collagen/Danilchik's Medium	454	435
Trunk neural crest cells 48h Collagen/Danilchik's Medium	316	375
Trunk neural crest cells 24h Collagen/Enriched Medium	415	346
Trunk neural crest cells 48h Collagen/Enriched Medium	216	225

Table 3.11: Outline for cell surface analysis. The total number of cells measured under each condition. ETOH = ethanol (alcohol)

All measurements were collected using the MetaMorph computer program. The data were exported to an SPSS (Statistical Package for the Social Sciences) spreadsheet to generate group statistics, analyze statistical assumptions, and perform the appropriate statistical analysis.

The differences in morphometric characteristics between control and ethanol treated cultures were tested for significance using the Wilcoxon rank sum test (Mann-Whitney U test) for nonparametric data. The same statistical analysis was then used to compare control groups to each other and experimental cultures to each other at the designated time intervals. The approximate normal derived Z (Z-scores) with a corresponding P-value less than or equal to 0.05 was considered to be significant. For each analysis, details are given with the relative Q-Q plots, Box-plots and Tables (Appendix C).

The above statistical analysis was supported and advised by the Training Consulting Centre, Department of Mathematics and Statistics at University of Alberta.

IV

CHAPTER FOUR

RESULTS

The effects of exposure of *Xenopus laevis* neural crest cells to low concentrations of ethanol *in vitro*.

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CHAPTER IV: RESULTS

4.1 Reproducibility

The results for intraobserver reproducibility are shown in Table 4.1. The mean error for different measurements ranged from a low of 0.8% (one value) to a high of 16.7% (one value), with the other values ranging between 3.4% and 5.2% (four values).

Table 4.1: Measurement Error. The measurement error for each parameter was calculated based on mean values.

	Mean (M)	**Average difference of mean (MD)**	**Mean error (MD/M) x 100%**
Number of individually emigrated cells	35.5	1.7 (± 0.7)	4.8%
Number of irregular cells	80.2	4.2 (± 2.1)	5.2%
Number of elongated cells	26.5	1.4 (± 1.2)	5.3%
Number of round cells	1.8	0.3 (± 0.3)	16.7%
Extant of migration	422.3 µm	3.3 µm (± 3.5)	0.8%
Size	2406.0 µm²	81.7 µm² (± 94.6)	3.4%

The value obtained for the number of round cells (16.7%) (Table 4.1) is high because the number of cells measured in this group was very small (under 10) subsequently making the results invalid. Therefore, caution should be used in accepting this value. Because the measurement errors for the rest of the parameters are approximately 5% or lower, these

results are satisfactory. In addition, based on the measurement of error for the extent of migration (0.8%), it is believed that the center of the explant was targeted consistently.

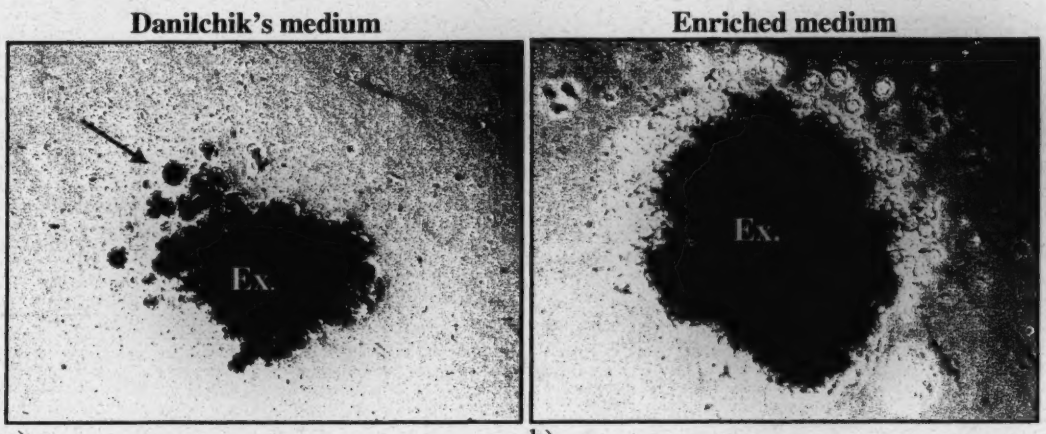
4.2 Pilot study-culturing cranial neural crest cells

Background

Past work from this laboratory has shown that *Xenopus* trunk neural crest will attach to and migrate on collagen or fibronectin[140]. The standard laboratory trunk culture conditions use collagen as the substrate and fibronectin in the medium[140]. However, attempts to use these same two substrates with cranial cultures failed as no attachment was attained (Milos, personal communication).

Since the purpose of this investigation was to observe the effects of alcohol on both cranial and trunk neural crest, it was essential to be able to culture cranial neural crest. Collagen was tested as a substrate and was found to be unsuitable (Fig. 4.2.1a, b), as cranial neural crest cells remained clustered and unattached. Approximately 80 cultures were tested with identical results in each.

Figure 4.2.1: Cranial neural crest cell behavior on Collagen at 3h post explant planting.



Legend for Figure 4.2.1a, b: Cranial neural crest cells do not attach or migrate on collagen in either Danilchik's (a) or enriched medium (b). Small numbers of round cells are observed at the explant peripheries (black arrow). At higher magnification cellular debris is also observed near the explant (red arrow). Ex. = cranial neural crest explant. (a) 25X, (b) 100X

On fibronectin as a substrate, cranial neural crest initially formed an explant with smooth outlines (Figure 4.2.2.a). By 1h the explant had usually attached to the substrate and cell migration was initiated. The cells began to form a dense cohesive epithelial sheet, consequently increasing the explant's surface area. The cells at the periphery of the explant extended wide, highly-branched protrusions in various directions around the

explant (Figure 4.2.2.b). By 2h the process of outgrowth was well underway as many more cells had spread out from the explant. At this time the epithelial sheet began to divide into separate sheets from which individual cells appeared to be breaking away and traveling in distinct directions (Figure 4.2.2.c). By 3h the separate streams of cells themselves had broken up and even more individual cells were present on the floor of the culture dish. The single cells had a wandering, fibroblastic-like morphology (Figure 4.2.2.d). However, this culture morphology was not maintained. By 5h the cells began to detach and by 10h all of them were found to be round and floating free in the medium.

These findings are consistent with Alfandari et al[5]. Thus, in culture, cranial neural crest cells, can attach to fibronectin in a simple medium, acquire motile properties, and embark upon extensive migration. This was observed at physiological pH (in the pilot study) as well as at pH 8.4[5].

Legend for Figure 4.2.2a, b, c, and d: These images show cranial neural crest explants cultured on fibronectin over a period of 3h. a) an explant with smooth peripheries (arrow), b) epithelial sheets are beginning to form, cells extend wide protrusions into the substrate (arrow), c) two separate groups of cells still contained within two epithelial sheets but cells are starting to break away, d) the epithelial sheets are breaking up into individual cells (arrow). Phase Contrast Microscopy.

Figure 4.2.2a: Cranial neural crest explants cultured on Fibronectin for 0h.

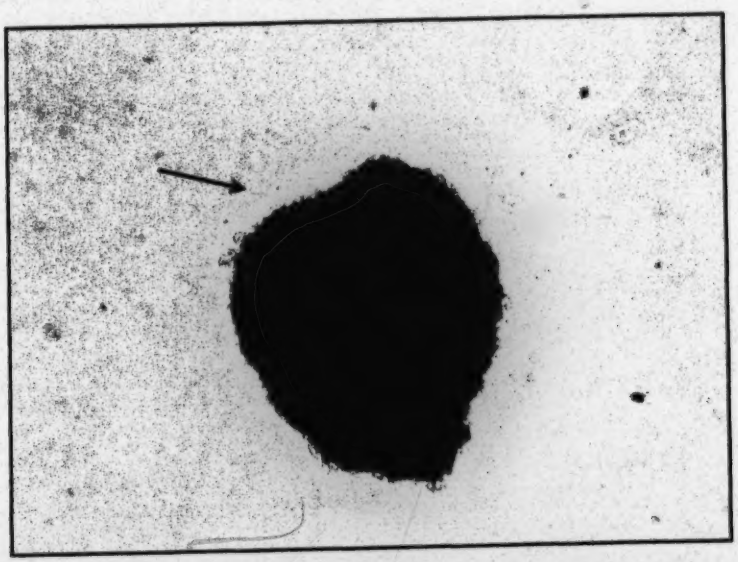


Figure 4.2.2b: Cranial neural crest explants cultured on Fibronectin for 1h.

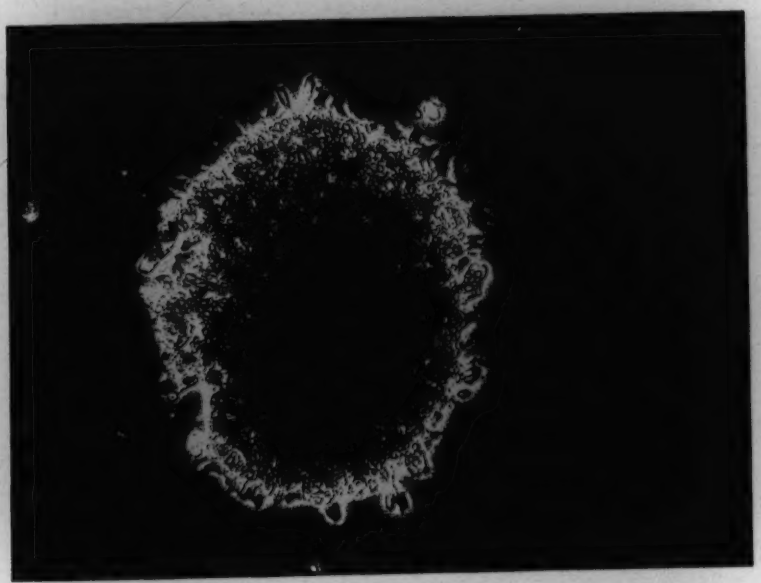


Figure 4.2.2c: Cranial neural crest explants cultured on Fibronectin for 2h.

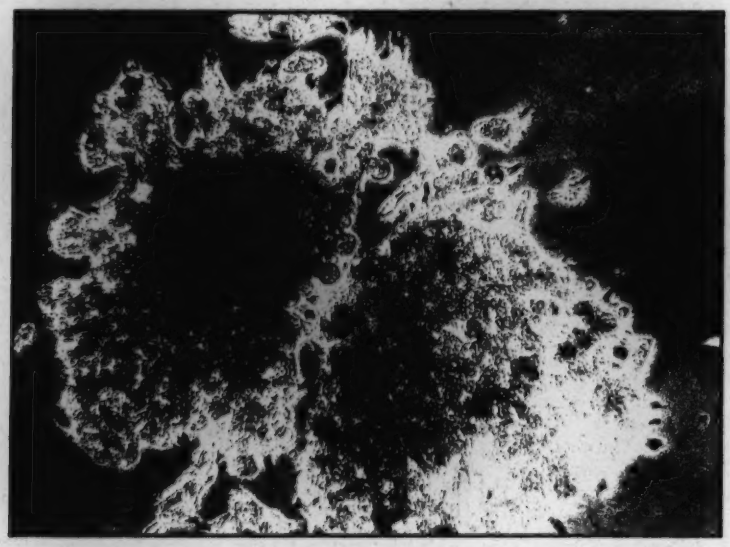
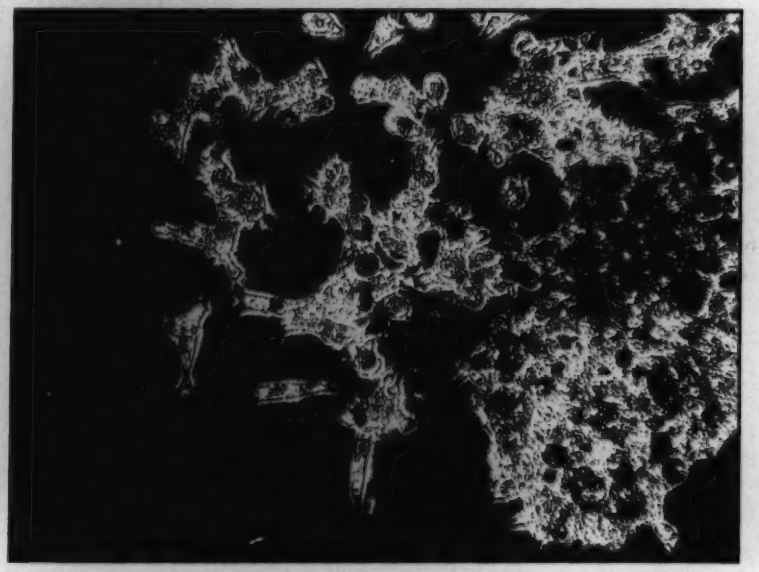


Figure 4.2.2d: Cranial neural crest explants cultured on Fibronectin for 3h.



4.3 The Effects of 0.05% Alcohol on Cranial Neural Crest Cells in Culture

4.3.1 General description of alcohol treated cultures

Cranial neural crest cells colonized the substrate in both control and alcohol-treated cultures, first as sheets and then as individual cells of migratory morphology. However, there were differences between control and experimental cultures (Figure 4.3.1). At 3h in the control cultures, some cranial neural crest cells were still contained in epithelial sheets, but many individual cells were beginning to separate. At 3h in the alcohol-treated group, the cells were migrating almost solely in epithelial sheets, and only very few individual cells had started to separate (Figure 4.3.1.a, c). However, in the next two hours the difference between the control and alcohol-treated groups was not as apparent because more individual cranial neural crest cells were observed on the floor of the culture dish in the alcohol-treated group, although many cells were still migrating in epithelial sheets (Figure 4.3.1.b, d).

Thus, the appearance of individual cells is delayed with alcohol treatment. This is further studied in the next section.

Legend for Figure 4.3.1a, b, c, and d: a) At 3h, in the control group many individual cranial neural crest cells are separating from the epithelial sheet b) At 5h, in the control cultures only individual cells are present in the culture dish. c) At 3h, alcohol-treated cranial neural crest cells remain in cohesive epithelial sheets. d) At 5h, in alcohol-treated cultures, epithelial sheets begin to break up and individual cells are observed in the culture dish. Black arrow = elongated cells, white arrow = irregular cells, orange arrow = round cells, black star = epithelial sheet. Ex. = Explant. Phase contrast microscopy, Bar 100μm

Figure 4.3.1.a: Control cranial neural crest cultured for 3h

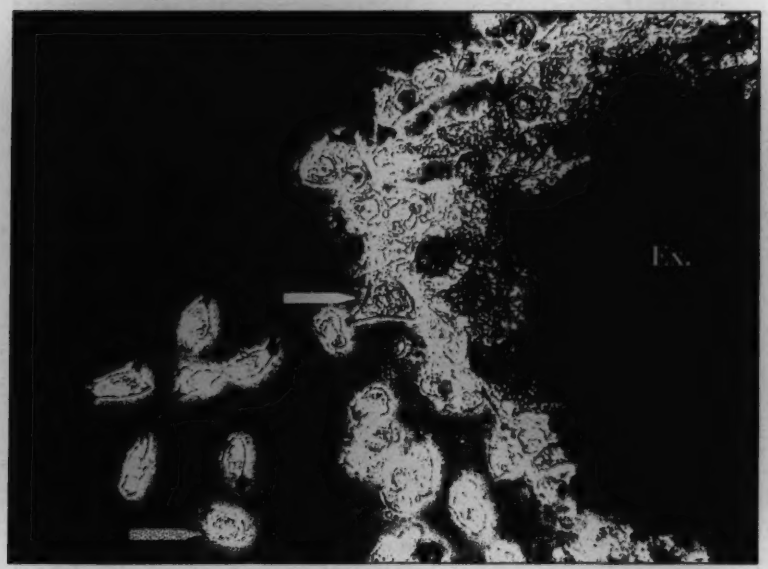
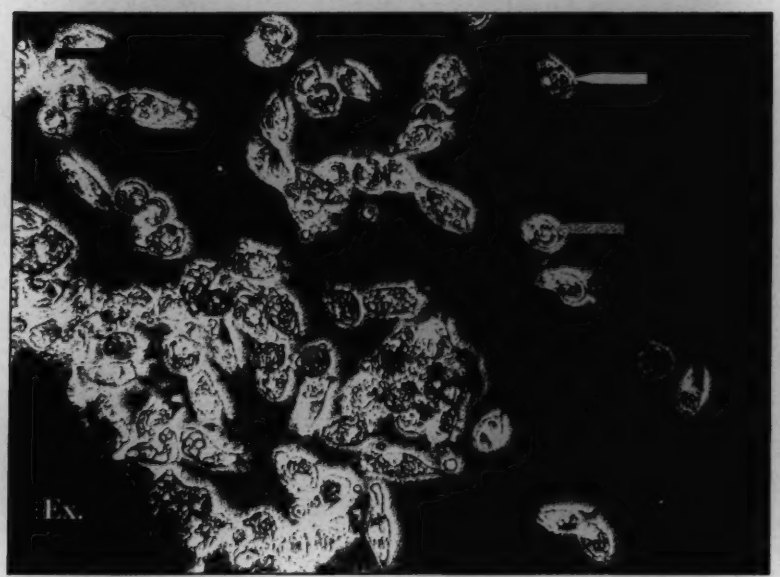


Figure 4.3.1.b: Control cranial neural crest cultured for 5h



The effects of exposure of *Xenopus laevis* neural crest cells to low concentrations of ethanol *in vitro*.

Figure 4.3.1.c: Experimental cranial neural crest cultured for 3h

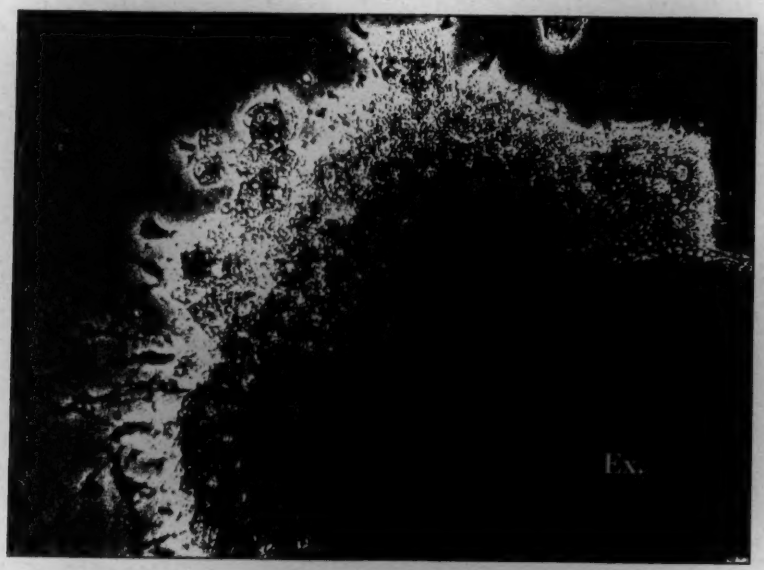
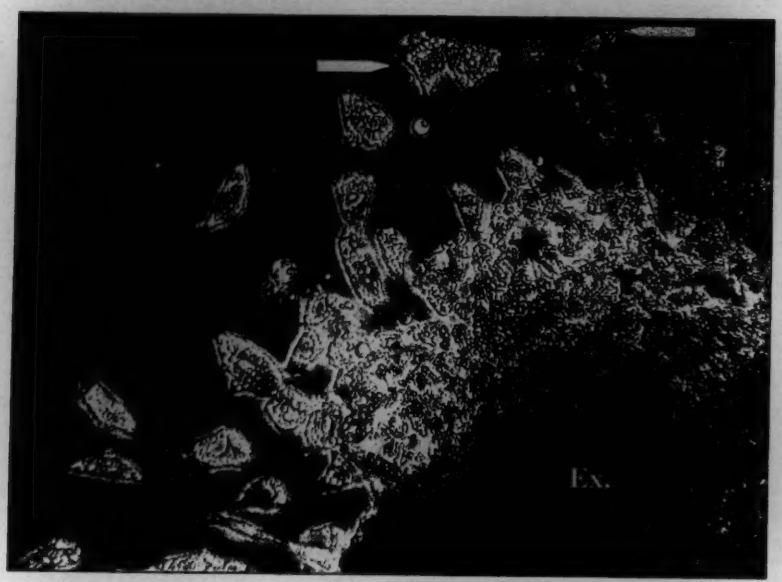


Figure 4.3.1.d: Experimental cranial neural crest cultured for 5h



The effects of exposure of *Xenopus laevis* neural crest cells to low concentrations of ethanol *in vitro*.

4.3.2 Cell counts

At 3h in the control cultures, cranial neural crest cells were migrating in epithelial sheets, but many individual cells were beginning to separate. On average there were 35 individual cells present in control cultures at this time. In the alcohol-treated group, the average number of individual cells dropped to 8 cells per culture, a 77% decrease on average ($p \leq 0.05$, Table 4.3.2, and Appendix C, Section 1.1.1).

By 5h the number of individual cells in alcohol-treated cultures increased significantly to 27 on average ($p \leq 0.05$, Table 4.3.2, and Appendix C, Section 1.1.2), such that there was no longer a significant difference between the control and alcohol-treated groups (Table 4.3.2, and Appendix C, Section 1.1.1).

Thus, alcohol treatment correlates with a lower number of individually emigrated cranial neural crest cells at 3h.

Legend for Table 4.3.2: This table shows the effects of 0.05% alcohol on cranial neural crest cell counts at 3 and 5h post explant planting. Averages represent estimates by visual inspection of 15 explants per treatment under 25X. Explants with epithelial sheets were seen significantly more often in experimental cultures. Cells in epithelial sheets were excluded from this analysis. (*) Significantly different from controls, (**) significantly different from 3h intervals, $p \leq 0.05$, by Wilcoxon Rank Sum test. Note: Values in the text are rounded to the nearest whole number. ETOH = ethanol (alcohol).

Page

Table 4.3.2: The effects of 0.05% alcohol on cranial neural crest cell counts at 3 and 5h post explant planting.

Cranial neural crest cells	Control 3h	ETOH 3h	Control 5h	ETOH 5h
Average# individually emigrated cells	34.80 (± 32.76)	8.33 (± 6.09) *	54.60 (± 43.15)	26.47 (± 14.36)**
% and averages of cells of different shapes				
irregular	57% (19.80, ±18.19)	78% (6.53, ±5.78)	64% (34.67, ±32.18)	76% (20.20,±15.03)**
elongated	21% (7.40, ±7.74)	14% (1.13, ±2.17) *	18% (9.93, ±7.21)	13% (3.47, ±4.45) *
round	22% (7.60, ±15.08)	8% (0.67, ±1.40) *	18% (10.00, ±14.27)	11% (2.80, ±6.22) *

The effects of exposure of *Xenopus laevis* neural crest cells to low concentrations of ethanol *in vitro*.

4.3.3 Cell Shapes

To determine if cell morphology changes with exposure to alcohol, cell shapes were examined. Even though cell concentration was initially high around the explant, three distinct cell shapes were identified: irregular, elongated and round (see Materials and Methods). As the cells separated, it was easier to distinguish cell shapes.

4.3.3.1 Proportions of cells in different shape categories

Irregular, flat cells with multiple extensions were in the majority under both conditions at both times (Table 4.3.2). At 3h, under control conditions 57% of the cells were irregular, while 78% of the cells were irregular in alcohol-treated cultures. At 5h, 68% of the cells in control cultures were irregular, while 76% of the cells maintained this shape in alcohol-treated groups. Elongated (bipolar with flattened leading lamellipodia), and round cells (without leading cytoplasmic projections) were also observed under both conditions. At 3h, in control cultures, 21% of the cells were elongated and 22% were round. At 3h, in alcohol-treated cultures, 14% of the cells maintained an elongated shape, while 8% were round. At 5h, in control cultures, 18% of the cells were elongated. This percentage was the same for the round cells. In alcohol-treated cultures 13% of the cells were elongated, while 11% of the cells maintained a round shape.

Thus, alcohol treatment appears not to affect the proportions of cells in different shape categories.

4.3.3.2 Counts of cells in different shape categories

Alcohol did not significantly change the number of irregular cells at the 3 or 5h intervals (Table 4.3.2, Appendix C, Section 1.2.1). An average of 20 irregular cells was present in control cultures at 3h, while in alcohol-treated cultures this value was 7 (Table 4.3.2). At 5h, the average number of irregular cells was 35 and 20 in control and alcohol-treated cultures respectively (Table 4.3.2).

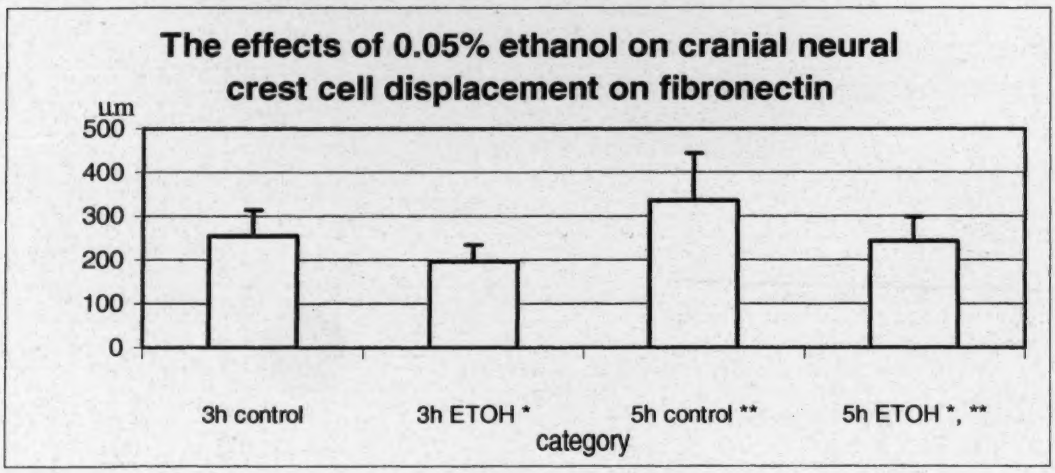
Alcohol did, however, significantly decrease the number of elongated and round cells at both intervals ($p \leq 0.05$, Table 4.3.2, Appendix C, Section 1.2.1). At 3h, there were on average 7 elongated cells present in control cultures, while in alcohol-treated cultures this value dropped to 1 (Table 4.3.2). By 5h, the number of elongated cells in control cultures was 10 while in alcohol-treated cultures this value was 3 (Table 4.3.2). At 3h, the number of round cells in control cultures was 8 but only 1 in the alcohol treated group (Table 4.3.2). At 5h, the average number of round cells in control cultures was 10 but only 3 in alcohol-treated cultures (Table 4.3.2).

In conclusion, alcohol treatment significantly affects the shape of cranial neural crest cells, as in the case of elongated and round cells. At 3h the number of elongated cells is lower by 85% while the number of round cells is lower by 91%. At 5h, the number of elongated cells is still lower by 65% and the number of round cells is still lower by 72%. This finding is not surprising since no significant increase was observed in the number of elongated and round cells in either group from 3 to 5h (Table.4.3.2, Appendix C, Section 1.2.2).

4.3.4 Distance moved by cranial neural crest cells

To determine whether cranial neural crest cell migration is affected by alcohol, the extent of migration of the outermost cells in both control and alcohol-treated cultures was also measured. By 3h the displacement distance in controls ranged from 164-344μm with an average of 255μm (Figure 4.3.4). This suggests a displacement rate of approximately 54-115μm/h, assuming that the cells travel at a constant rate in a straight line. In contrast, alcohol-treated cells moved 139-252μm by 3h, an average of 197 μm, which is significantly less than controls by 23% ($p \leq 0.05$, Figure 4.3.4, and Appendix C, Section 1.3.1). This displacement rate ranged from 46 -84μm/h (using similar assumptions).

Figure 4.3.4: Bar graph of cranial neural crest cell displacement.



Legend for Figure 4.3.4: The plotted values are means with one standard deviation bars for each group. (*) Significantly different from control, (**) significantly different from 3h group in each category, $p \leq 0.05$, by Wilcoxon Rank Sum test. Note: Values in the text are rounded to the nearest whole number. ETOH = ethanol (alcohol).

Both groups showed a significant increase in displacement by 5h, ($p \leq 0.05$, Figure 4.3.4, Appendix C, Section 1.3.2). The cells in the control group dispersed on average 336µm (25% more) while the cells in the alcohol-treated group dispersed an average of 243µm (19% more). The range of values in the control group was 215-499µm (a rate of 43-100µm/h) while the cells in the experimental group dispersed between 157-326µm (a rate of 31-65µm/h). Interestingly, at 5h the average distance traveled by the alcohol-

treated group was still about 28% less than the average distance traveled by the cells of the control group ($p \leq 0.05$, Figure 4.3.4, Appendix C, Section 1.3.1).

Thus, alcohol also has negative effects on cranial neural crest cell displacement. This effect occurs for cells traveling individually and at the edges of epithelial sheets.

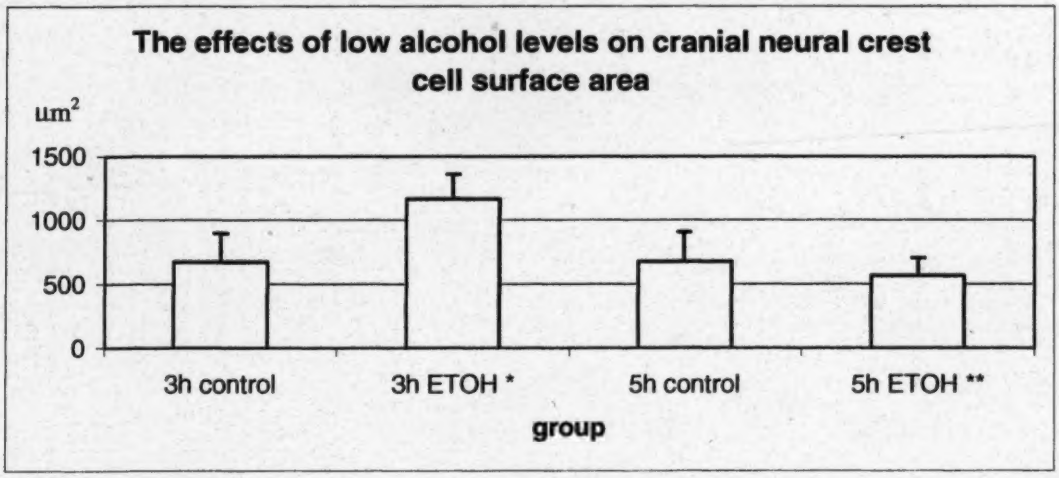
4.3.5 Cell surface area (spread)

This study also determined if the surface area (spread) of cranial neural crest cells is affected by alcohol. There was a significant difference in cell surface area at 3h ($p \leq 0.05$, Figure 4.3.5, Appendix C, Section 1.4.1). On average, control cells had a mean surface area of 674μm^2 while alcohol-treated cells had an average value of 1162μm^2. Thus, the alcohol-treated cells were almost twice as large as the controls. At 5h the cell surface area in the alcohol-treated group dropped significantly to 567μm^2 ($p \leq 0.05$, Figure 4.3.5, Appendix C, Section 1.4.2), to the point where it was no longer statistically different from controls (Figure 4.3.5, Appendix B, Section 1.4.1).

As a note of caution, because at 3h alcohol-treated cranial neural crest cells migrated mainly in epithelial sheets, the surface area of only 37 individual cells from the experimental cultures was compared to the surface area of 271 cells from the control cultures [for cell selection see Materials and Methods].

Thus, overall alcohol affects the cell surface area, causing an increase in spreading at 3h of culture.

Figure 4.3.5: Bar graph of cranial neural crest cell surface area.



Legend for Figure 4.3.5: On fibronectin, the average size of ethanol-treated cranial neural crest cells is greater only at 3h. The average size of alcohol-treated cells decreases with time. (*) Significantly different from controls, (**) significantly different between intervals. Wilcoxon rank sum test, $p \leq 0.05$). The plotted values are means and the bars represent one standard deviation. Note: Values in the text are rounded to the nearest whole number. ETOH = ethanol (alcohol).

4.4 The Effects of 0.05% Alcohol on Trunk Neural Crest Cells in Culture

For the purpose of comparison to cranial neural crest, trunk neural crest cells were exposed to alcohol *in vitro*. Past work from this laboratory has shown that trunk neural crest cells migrate on both fibronectin and collagen, in both defined media with and without serum[140]. In a medium containing serum, most cells differentiate into melanophores, while in a serum-free medium neurite cell extensions grow out of the explant in addition to melanophores[140].

4.4.1 General description of trunk neural crest cultures

In control cultures, the growing conditions affected the migration pattern of trunk neural crest cells similarly, at both 24 and 48h (Figure 4.4.1.1a, b, c, d, e, and f).

In Danilchik's medium (on either fibronectin or collagen as a substrate), the majority of the cells appeared to migrate individually (although upon visual inspection the cells were more dispersed on fibronectin than on collagen) (Figure 4.4.1.1.a, b, c, d). Many of the cells had a fibroblast-like morphology (Figure 4.4.1.1.a, b, c, d). In the enriched medium, however, although individual cells were observed, the majority of the cells maintained closer cell-to-cell interactions, were overlapping, and remained closer to the explant (Figure 4.4.1.1.e, f). At 48h, the cells in the enriched medium had an epithelial-like morphology.

Similar observations were made for alcohol-treated cultures. As in controls, a significant number of individual trunk neural crest cells migrated from the explant by 24h under all growing conditions. The majority of the cells migrated individually in Danilchik's medium while in the enriched medium the cells overlapped and were found in close proximity to the explant (Figure 4.4.1.2.a, c, e). Unlike cranial neural crest cells (which, at 3h, were not migrating individually), individual cells were observed in the alcohol-treated trunk neural crest cells under all of the growing conditions.

Thus, low alcohol levels do not affect trunk neural crest differentiation under any growing conditions during the tested intervals. At both the 24 and 48h in the Danilchik's medium, neurites were directly recognized by the presence of long cones, and in the enriched medium pigment cells were recognized by the presence of pigment granules (Figure 4.4.1.2).

Legend for Figure 4.4.1.1a, b, c, d, e and f: This figure shows control trunk neural crest explants under different growing conditions. Individual cells of different shapes are observed under all growing conditions. Pigment cells are noted in enriched medium while neurites are visible in Danilchik's medium. Cells in close proximity to the explant are only noted in enriched medium. Black arrow: pigment cells; Red arrow: neurites; Black pointer: elongated cells, White pointer: irregular cells, Orange pointer: round cells; Star: cells in close proximity to the explant. Ex. = Explant. Phase contrast microscopy, Bar 100µm

Figure 4.4.1.1a: Control trunk neural crest cultured on Fibronectin in Danilchik's Medium for 24h

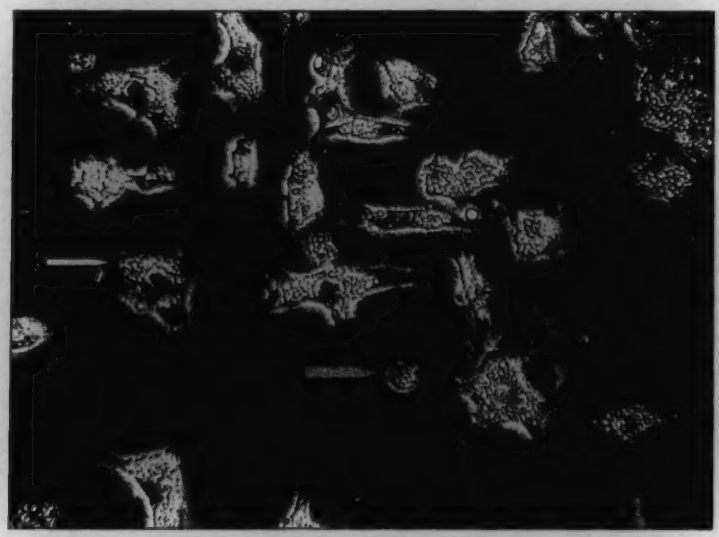
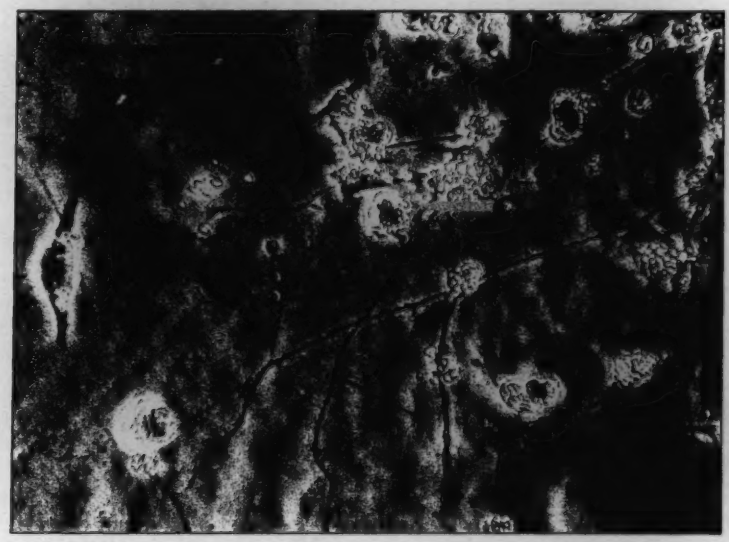


Figure 4.4.1.1b: Control trunk neural crest cultured on Fibronectin in Danilchik's Medium for 48h



The effects of exposure of *Xenopus laevis* neural crest cells to low concentrations of ethanol *in vitro*.

Figure 4.4.1.1c: Control trunk neural crest cultured on Collagen in Danilchik's Medium for 24h

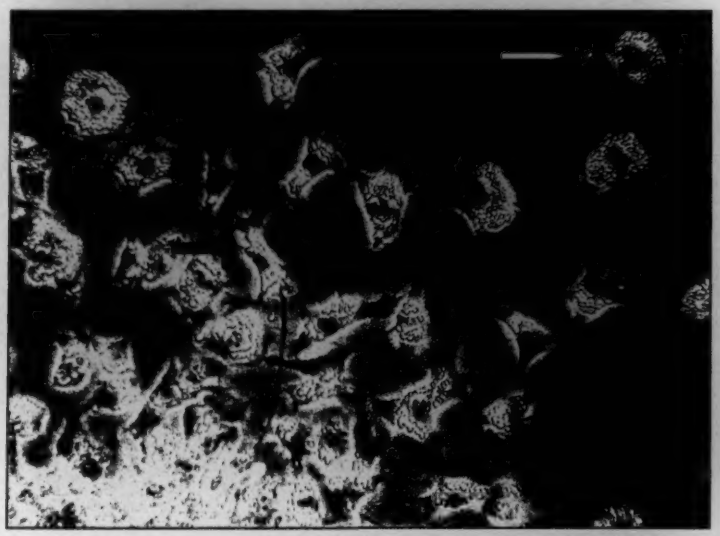


Figure 4.4.1.1d: Control trunk neural crest cultured on Collagen in Danilchik's Medium for 48h

Figure 4.4.1.1e: Control trunk neural crest cultured on Collagen in an Enriched Medium for 24h

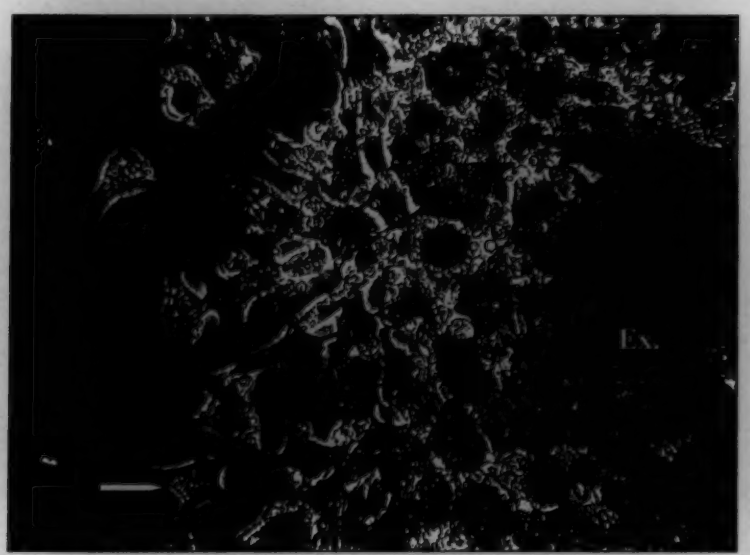
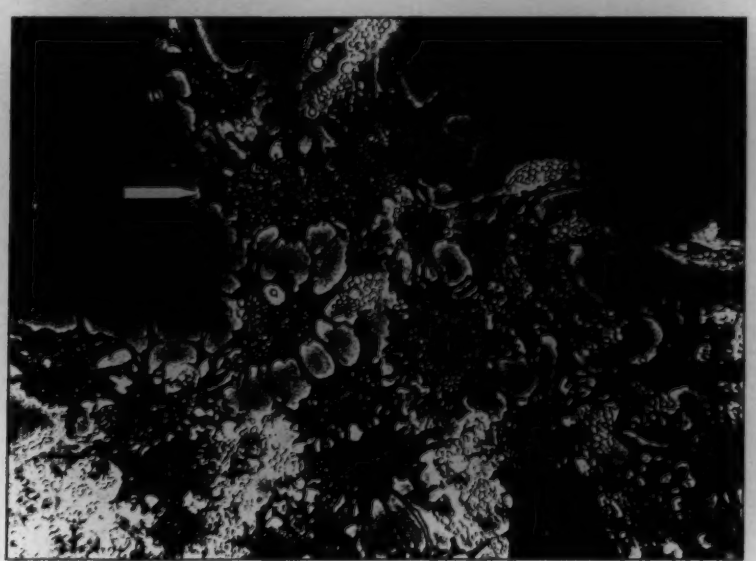


Figure 4.4.1.1f: Control trunk neural crest cultured on Collagen in an Enriched Medium for 48h



The effects of exposure of *Xenopus laevis* neural crest cells to low concentrations of ethanol *in vitro*.

Legend for Figure 4.4.1.2a, b, c, d, e, and f: This figure shows alcohol-treated trunk neural crest explants under different growing conditions. As in controls (above), individual cells of different shapes are observed under all growing condition. Pigment cells and neurites are visible in enriched and Danilchik's medium respectively. As in controls (above), cells in close proximity to the explant are also noted only in enriched medium. Black arrow: pigment cells; Red arrow: neurites; Black pointer: elongated cells, White pointer: irregular cells, Orange pointer: round cells; Star: cells in close proximity to the explant. Ex.= Explant. Phase contrast microscopy, Bar 100µm.

Figure 4.4.1.2.a: Experimental trunk neural crest cultured on Fibronectin in Danilchik's Medium for 24h

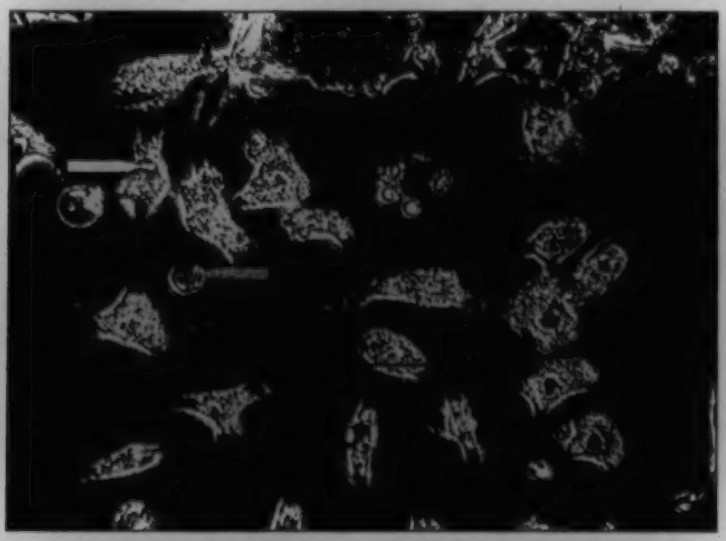


Figure 4.4.1.2.b: Experimental trunk neural crest cultured on Fibronectin in Danilchik's Medium for 48h

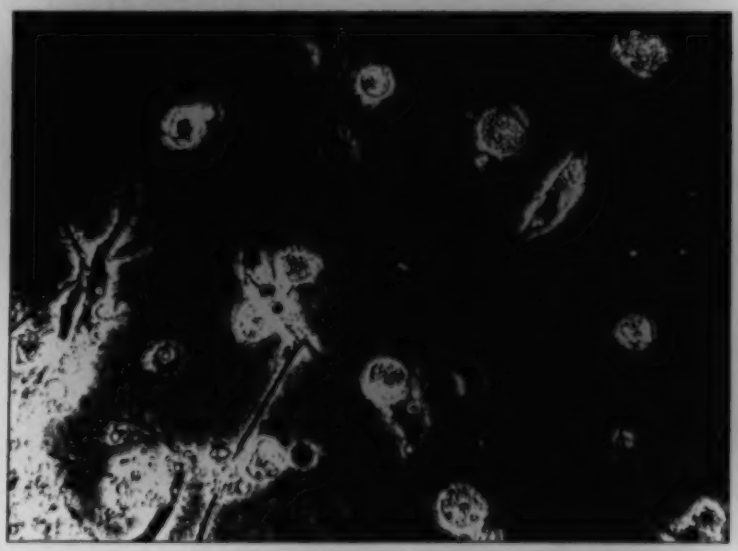


Figure 4.4.1.2.c: Experimental trunk neural crest cultured on Collagen in Danilchik's Medium for 24h

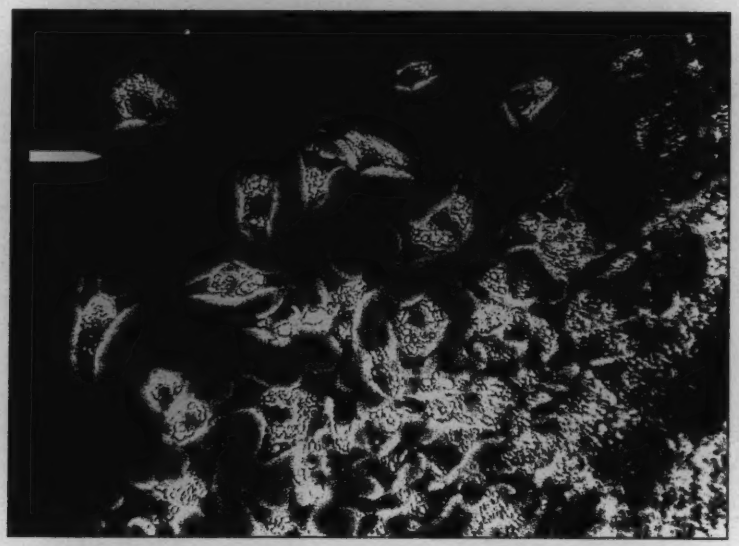
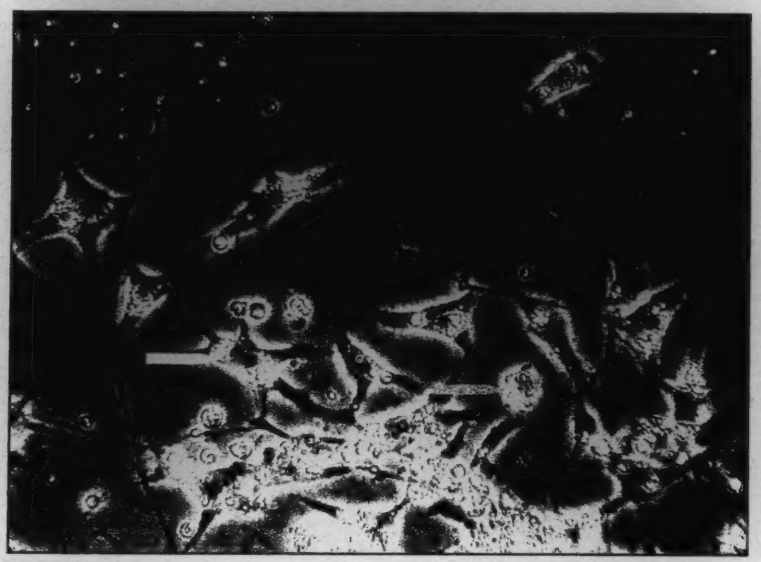


Figure 4.4.1.2.d: Experimental trunk neural crest cultured on Collagen in Danilchik's Medium for 48h



The effects of exposure of *Xenopus laevis* neural crest cells to low concentrations of ethanol *in vitro*.

Figure 4.4.1.2.e: Experimental trunk neural crest cultured on Collagen in an Enriched Medium for 24h

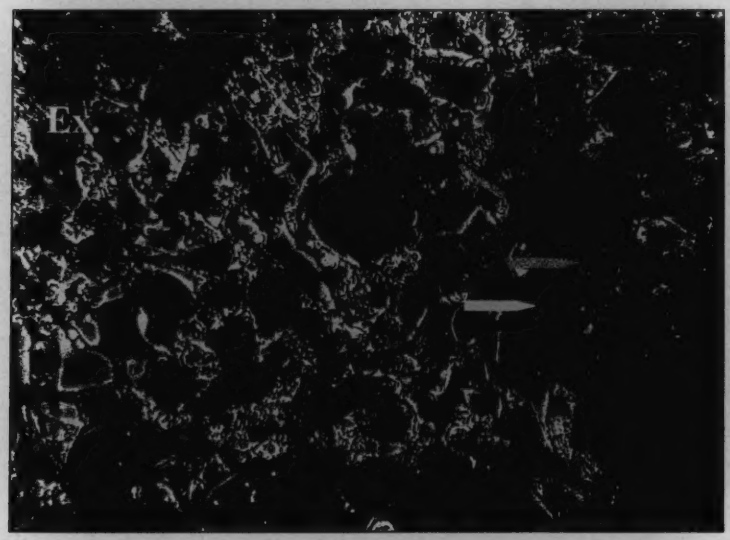
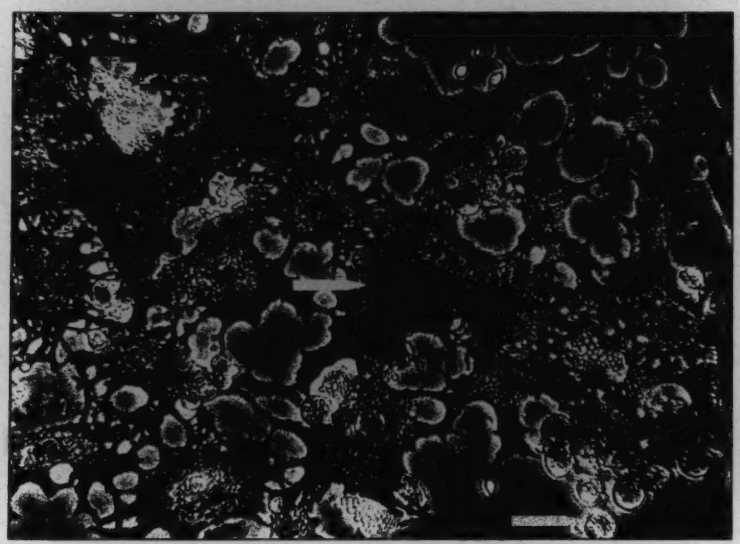


Figure 4.4.1.2.f: Experimental trunk neural crest cultured on Collagen in an Enriched Medium for 48h



4.4.2 Cell counts

Individual cells in both control and alcohol-treated groups were counted at both times. In contrast to cranial neural crest cells at 3h, alcohol levels of 0.05% did not affect the number of individually emigrated cells at 24h under any of the three tested conditions (Table 4.4.2. a, b, c, Appendix C, Section 2.1.1, 3.1.1, 4.1.1). On fibronectin there were on average 83 individual cells present in the control cultures, and in the alcohol-treated cultures this number was almost identical (an average of 85 cells per culture). On collagen in Danilchik's medium, the average number of individual cells in control cultures was 76, while for alcohol-treated cultures it was 86. Similarly, on collagen in an enriched medium the average number of individual cells in controls was 55 while in the alcohol-treated group it was 42.

From 24 to 48h, there were differences observed in the cell counts for the various growing conditions and treatments. On fibronectin, the cell count in both the control and experimental groups decreased significantly from 83 to 52 cells (by 37%) and 85 to 62 cells (by 27%) respectively ($p \leq 0.05$, Table 4.4.2.a, Appendix C, Section 2.1.2). On collagen in Danilchik's medium, only the cell count in the experimental group decreased significantly, from 86 to 59 cells (by 33%) ($p \leq 0.05$, Table 4.4.2.b, Appendix C, Section 3.1.2). These decreases might be explained by detachment because the number of round cells quintupled from 24 to 48h (Appendix C, Section 3.2.2). Some of the round cells

might also have drifted away from the explants. Interestingly, although the average cell count for the cultures growing on collagen in enriched medium remained constant from 24 to 48h (56 cells on average at both times), the cell count in the experimental group actually increased by 25% [but this change was not statistically significant (Table 4.4.2.c, Appendix C, Section 4.1.2)].

Therefore, in Danilchik's medium on fibronectin, there is a significant drop in cell count at 48h in both the presence and absence of alcohol. Enriched medium appears to be able to protect against the drop in cell number in both the presence and absence of alcohol.

None of the changes from 24 to 48h were enough to produce a significant difference in the cell counts between the control and experimental groups at 48h (Table 4.4.2.a, b, c, Appendix C, Section 2.1.1, 3.1.1, 4.1.1), results that were similar to cranial neural crest cell experiments at 5h. On fibronectin, an average of 52 individual cells was present in the control cultures, while in the alcohol-treated group this number was 62 (a difference of 16%). On collagen in a simple medium, there was an average of 53 and 59 individual cells per culture in the control and alcohol-treated groups respectively (a difference of 10%). On collagen in enriched medium the numbers were 56 for the control and 53 for the alcohol-treated groups (a difference of 5%).

Thus, alcohol does not affect trunk neural crest cell numbers in culture at either interval under all tested conditions.

Legend for Table 4.4.2a, b, and c: This table shows the effect of 0.05% alcohol on trunk neural crest cell counts. Averages represent estimates by visual inspection under 25X. Cells that were incorporated into dense, multi-layered sheet were excluded from this analysis. a) Fibronectin/Danilchik's Medium, b) Collagen/Danilchik's Medium, c) Collagen/Enriched Medium. (*) Significantly different from controls, (**) significantly different from 24h intervals, $p \leq 0.05$, Wilcoxon rank sum test. Note: Values in the text are rounded to the nearest whole number. ETOH = ethanol (alcohol), TNCC = Trunk Neural Crest Cells, ave. = average

Table 4.4.2a: The effects of 0.05% alcohol on trunk neural crest cell counts grown on Fibronectin in Danilchik's Medium at 24 and 48h.

TNCC Fibronectin Danilchik's Medium	Control 24h	ETOH 24h	Control 48h	ETOH 48h
Average # individually emigrated cells	83. 40 (±52.56)	84.60 (±21.86)	51.93 (±32.36)**	61.87 (±14.91)**
% and ave. of cells of different shapes				
irregular	73 % (61.07, ±44.45)	70% (59.47, ±19.66)	20% (10.13, ±11.89)**	23% (14.27, ±5.72)**
elongated	23% (19.27, ±18.59)	22% (18.80, ±11.15)	31% (16.33, ±16.09)	20% (12.40, ±9.52)
round	4% (3.07, ±3.15)	8% (6.33, ±4.10) *	49% (25.47, ±19.23)**	57% (35.20, ±18.52)**

The effects of exposure of *Xenopus laevis* neural crest cells to low concentrations of ethanol *in vitro*.

Table 4.4.2.b: The effects of 0.05% alcohol on trunk neural crest cell counts grown on Collagen in Danilchik's Medium at 24 and 48h.

TNCC Collagen Danilchik's Medium	Control 24h	ETOH 24h	Control 48h	ETOH 48h
Average # individually emigrated cells	75.87 (± 38.03)	85.53 (± 37.85)	53.20 (±24.38)	58.93 (±24.05)**
% and ave. of cells of different shapes				
irregular	87 % (58.20, ±45.13)	77% (74.20, ±47.16)	60% (31.87, ±26.11)	58% (34.33, ±22.76)**
elongated	10% (10.80, ±12.48)	14% (8.53, ±14.88)	16% (8.67, ±10.38)	17% (9.80, ±8.95)
round	3% (6.87, ±9.78)	8% (2.8, ±3.80)	24% (12.67, ±9.71)**	25% (14.80, ±8.89)**

The effects of exposure of *Xenopus laevis* neural crest cells to low concentrations of ethanol *in vitro*.

Table 4.4.2.c: The effects of 0.05% alcohol on trunk neural crest cell counts grown on Collagen in an Enriched Medium at 24 and 48h.

TNCC Collagen Enriched Medium	Control 24h	ETOH 24h	Control 48h	ETOH 48h
Average # individually emigrated cells	55.46 (± 25.24)	41.47 (± 30.82)	56.27 (±27.94)	53.00 (±18.02)
% and ave. of cells of different shapes				
irregular	83% (46.40, ±25.67)	89% (37.00, ±30.18)	85% (47.93, ±26.72)	83% (43.93, ±17.59)
elongated	4% (2.40, ±1.92)	4% (1.8, ±2.46)	2% (0.93, ±1.87)**	1% (0.67, ±1.05)
round	13% (7.40, ±8.89)	6% (2.67, ±2.94)	13% (7.40, ±6.40)	16% (8.67, ±7.01)**

The effects of exposure of *Xenopus laevis* neural crest cells to low concentrations of ethanol *in vitro*.

Page

4.4.3 Cell shapes

To further investigate trunk neural crest cell responses in the presence of a low alcohol level, the cells were classified according to the three cell shapes discussed above.

4.4.3.1 Proportions of cells in different shape categories

Similar proportions of the different cell shapes were found in the control and experimental groups, under all conditions, at 24h and 48h (Table 4.4.2.a, b, c). In both Danilchik's medium and enriched medium at 24h, the majority of the cells in control and alcohol-treated cultures were of irregular shapes. On fibronectin the percentage of irregular cells was almost identical for the control and alcohol-treated groups; 73% for controls and 70% for the alcohol-treated group. On collagen, in Danilchik's medium, the percentage of irregular-shaped cells ranged from 77% to 87% for control and alcohol-treated groups respectively, and in enriched medium the percentage of irregular cells in control group was 83% while in alcohol-treated group it was 89%.

At 24h, elongated cells were also observed in similar proportions under all conditions. As with irregular cells, on fibronectin the proportion of elongated cells was almost identical for the control and alcohol-treated groups (22% and 23% respectively). On collagen in Danilchik's medium the proportions of elongated cells were also almost constant (10% and 14% for control and alcohol-treated group respectively). In enriched medium a similar trend was observed (4% for both groups), however the elongated cells

were the least common variety. In contrast, round cells were the least common cells in Danilchik's medium during this interval (they ranged from 3% in control cultures to 8% in alcohol-treated cultures).

At 48h, the proportions changed only in Danilchik's medium. On collagen, the irregular cells still remained as the majority in both groups (~60%). However on fibronectin the irregular cells appeared to be the least common ones, forming approximately 20% of the population (a decrease by 70% for both control and alcohol-treated groups). Furthermore, on fibronectin, the proportion of round cells increased by approximately 90% in both groups, making them the majority under both control and experimental conditions.

4.4.3.2 Counts of cells in different shape categories

Low levels of alcohol only changed the cell count significantly in one group; the round cells on fibronectin at 24h increased by 100% ($p \leq 0.05$, Table 4.4.2.a, Appendix C, Section 2.2.1). In the control group, on average 3 round cells were present while this number doubled in the alcohol-treated group. No significant differences were observed for any other cell shapes, under any other growing conditions, at either 24 or 48h (Table 4.4.2.b, c, Appendix C, Section 3.2.1, 4.2.1).

When contrasting the 24h and 48h measurements, in Danilchik's medium, the number of irregular cells in the experimental groups dropped significantly (on fibronectin

and collagen) from 24 to 48h, while the number of round cells increased significantly ($p \leq 0.05$, Table 4.1.2.a, b, Appendix C, Section 2.2.2, 3.2.2). On fibronectin, in the experimental group, the number of irregular cells dropped from 60 cells on average to 14 by 48h (a decrease of 84%). On collagen, the number of irregular cells in the experimental group dropped from 74 to 34 on average (a decrease of 54%). On fibronectin, the number of round cells in the experimental group increased from 6 to 35 by 48h (an increase of more than 80%). On collagen the number of round cells also increased by more than 80%. In the control group, the number of irregular cells also decreased significantly on fibronectin, from 61 to 10 on average, while the number of round cells increased significantly on both substrates, similar to the experimental groups (Table 4.1.2.a, b, Appendix C, Section 2.2.2, 3.2.2). In the control group, the number of round cells on fibronectin increased from 3 to 25 cells (an increase of almost 90%) and on collagen the number of round cells increased from 7 to 13 (an increase of almost 50%).

In an enriched medium, these changes were not observed. The numbers of irregular, elongated and round cells stayed the same from 24 to 48h, for both the control and alcohol-treated cultures (Table 4.1.1.c, Appendix C, Section 4.2.2). The explanation for this might be that, for trunk neural crest cells, Danilchik's medium does not support long-term growth in the same way as enriched medium.

4.4.4 Distances moved by trunk neural crest cells

Next the study examined the displacement distances of trunk neural crest cells in the presence and absence of alcohol. At 24h there was no significant difference between control and experimental trunk displacement distances under any growing conditions (Figure 4.4.4, Appendix C, Section 2.3.1, 3.3.1, 4.3.1). Assuming that the cells move in a straight line, on fibronectin control cells displaced an average of 506μm, with a range of 235-860μm (a rate of 10-36μm/h). In the alcohol-treated group the cells displaced an average of 599μm (a 16% increase) with a range of 325-768μm (a rate of 14-32μm/h). On collagen, in Danilchik's medium, the control group displaced an average of 371μm with a range of 111-588μm (a rate of 5-25μm/h). An almost identical displacement was observed in the alcohol-treated group [377μm with a range of 253-602μm (a rate of 11-25μm/h)]. On collagen, in enriched medium, control cells displaced an average of 363μm with a range of 246-475μm (a rate of 10-20μm/h). For alcohol-treated cells the value was 331μm (a 9% decrease) with a range of 227-478μm (a rate of 10-20μm/h).

At 48h there was no significant difference on collagen (Figure 4.4.4, Appendix C, Section 3.3.1, 4.3.1). In Danilchik's medium, cells from the control group displaced on average 346μm with a range of 114-551μm (a rate of 2-12μm/h). In the alcohol-treated group the cells displaced on average 358μm (a 3% increase) with a range of 192-564μm (a rate of 4-12μm/h). In enriched medium, the cells in the control group displaced on average 504μm with a range of 192-615μm (a rate of 4-13μm/h) and in the alcohol-

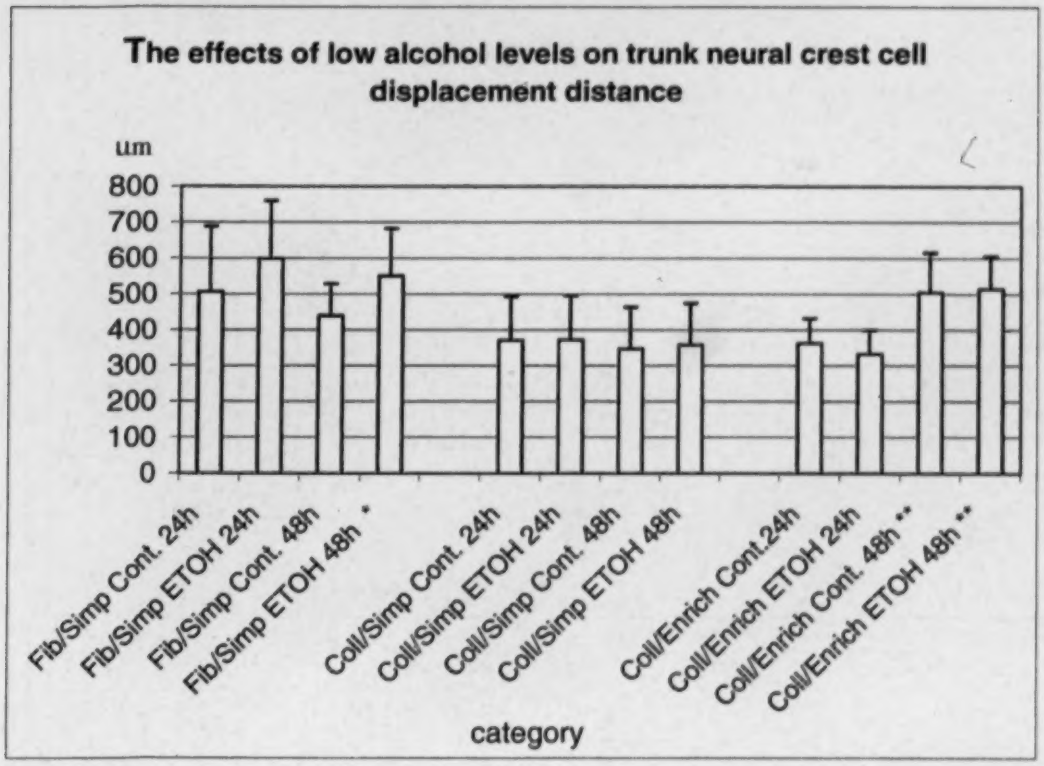
treated group the cells displaced an average of 513μm (a 2% increase) with a range of 227-478μm (a rate of 5-10μm/h).

However, on fibronectin trunk neural crest cell displacement was 20% higher (statistically significant) in the experimental group than in the control group ($p \leq 0.05$, Figure 4.4.4, Appendix C, Section 2.3.1). The most peripheral control trunk neural crest cells on fibronectin displaced an average of 439μm with a range of 261-567μm (a rate of 5-12μm/h) while the experimental cells displaced an average of 551μm with a range of 329-735μm (a rate of 7-15μm/h).

[Note that the similar increase of 20% was observed on fibronectin at 24h (as described above), but was not statistically significant because of the high standard deviation in both groups (183μm and 160μm for the control and alcohol-treated groups respectively) (Figure 4.4.4, Appendix C, Section 2.3.1)].

Thus, based on the results obtained from 48h cultures, alcohol enhances trunk neural crest cell migration on fibronectin.

Figure 4.4.4: Bar graph of trunk neural crest cell displacement.



Legend for Figure 4.4.4: Averages and standard deviation bars. (*) Significantly different from controls, (**) significantly different from the 24h group in each category, $p \leq 0.05$, Wilcoxon rank sum test. Note: Values in the text are rounded to the nearest whole number. ETOH = Ethanol (alcohol), Fib = Fibronectin, Simp = Simple (Danilchik's) medium, Coll = Collagen, Enrich = Enriched medium, Cont. = Controls

In addition, at 48h trunk neural crest cells in both control and experimental groups significantly increased their displacement only in an enriched medium (Figure 4.4.4). This suggested that an enriched medium was more capable of supporting long-term cultures than Danilchik's medium.

4.4.5 Cell surface area (spread)

Next, to determine whether alcohol changes trunk neural crest cell size (spread), this study examined the cell surface area of trunk neural crest cells cultured under different growing conditions.

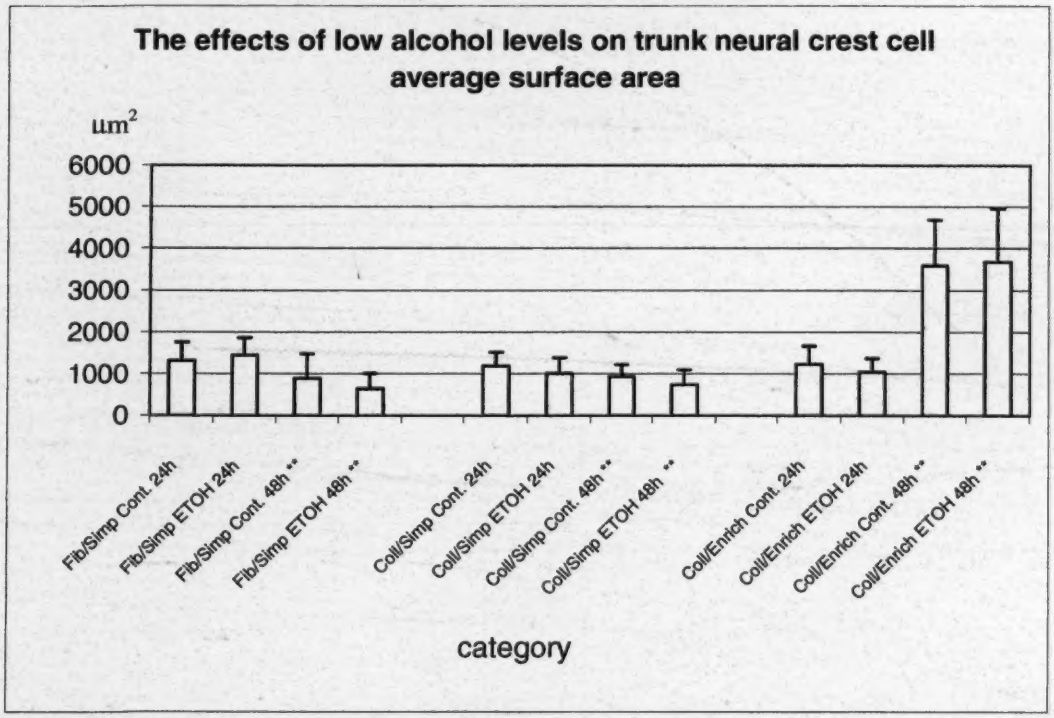
There was no significant difference in cell surface area between the control and experimental groups under any of the growing conditions at 24 or 48h (Figure 4.4.5, Appendix C, Section 2.4.1, 3.4.1, 4.4.1). On fibronectin at 24h, the average cell surface area of the control group was 1317μm^2, while the average for the alcohol-treated group was 1447μm^2 (a 9% increase). At 48h the average cell surface for controls was 890μm^2 while the average for the alcohol-treated group was 646μm^2 (a 27% decrease).

On collagen with Danilchik's medium the average cell surface area for controls was 362μm^2 and 332μm^2 for the alcohol-treated group at 24h (an 8% decrease). At 48h it was 504 and 514μm^2 for the control and alcohol-treated groups respectively (a 2% increase). On collagen in an enriched medium at 24h the average size was 1236μm^2 for controls and 1034μm^2 for the experimental group (a 16% decrease). At 48h the averages

were 3590μm² and 3674μm² for the control and experimental groups respectively (a 2% increase).

Cell size did change significantly, however, from 24 to 48h ($p \leq 0.05$, Figure 4.4.5, Appendix C, Section 2.4.2, 3.4.2). In Danilchik's medium on fibronectin, the average cell size decreased by approximately one-third for the control group (from 1316μm² to 890μm²) and by more than one-half for the experimental group (from 1446μm² to 645μm²). On collagen, for both groups, the average cell size decreased by approximately one-quarter. In the control group the average cell size decreased from 1189μm² to 1014μm² while in the alcohol treated group the average size decreased from 924μm² to 740μm². In enriched medium, the average cell size approximately tripled for both groups ($p \leq 0.05$, Figure 4.4.5, Appendix C, Section 4.4.2). The cell size in the control group increased from 1236μm² to 3590μm² while in the alcohol-treated group the size increased from 1033μm² to 3674μm².

Figure 4.4.5: Bar graph of trunk neural crest cell surface area.



Legend for Figure 4.4.5: Alcohol did not affect trunk NCC surface area. Columns represent means and bars represent one standard deviation. (**) Significantly different from the 24h group in each category, $p \leq 0.05$, Wilcoxon rank sum test. Note: Values in the text are rounded to the nearest whole number. ETOH = Ethanol (alcohol), Fib = Fibronectin, Simp = Simple (Danilchik's) medium, Coll = Collagen, Enriched = Enriched medium, Cont.= Controls

In summary, alcohol does not affect average trunk neural crest cell surface area.

V

CHAPTER FIVE

DISCUSSION

CHAPTER V: DISCUSSION

5.1 Accomplishments

This study accomplished four major tasks:

1. culturing of cranial neural crest cells;
2. culturing of trunk neural crest cells in saline;
3. testing the effects of a low concentration of alcohol (equivalent to one standard drink) on both cranial and trunk neural crest cells and establishing that they respond differently to alcohol; and
4. establishing a simple, reproducible, reliable system for quantitative analysis of the *in vitro* investigations.

The first accomplishment (successful culturing of *X. laevis* cranial neural crest cells) is a step forward because previous attempts to grow cranial neural crest cells *in vitro* have not been successful. An extensive literature search was performed to give some insights into the conditions required for *X. laevis* cranial neural crest cells to grow in culture. A recent study by Alfandari et al[5] demonstrated that in the absence of other tissues, in Danilchik's medium containing BSA at pH 8.4, *Xenopus* cranial neural crest cells extracted from embryos at stage 17 attach and migrate on fibronectin. In the present study, Alfandari's experiment was performed with some modifications to see if the same results could be obtained in this laboratory. The pH was adjusted to 7.4 (to imitate normal

physiological conditions), BSA was omitted (inhibitors of nonspecific binding were not a necessary component in this investigation), and cranial neural crest cells were extracted from embryos at stages 19-21 (to capture the initial period of neural crest cell migration). Under these conditions neural crest cells attach to fibronectin, acquire motile properties, and migrate extensively. Thereby this study provides s very effective technique for testing the effects of alcohol on early migrating neural crest cells which can offer a better understanding of the responses to alcohol at the cellular level.

Furthermore, this *in vitro* investigation demonstrates that, under these conditions, cranial neural crest cells behave in a similar manner as *in vivo*. Initially they migrate in dense, cohesive sheets (Figure 4.2.b) just as they do *in vivo*[109]. Subsequently, the explants begin to divide into separate entities (Figure 4.2.c) and the migrating cell sheets appear to travel in distinct streams, again as seen *in vivo*[109]. The separate streams of epithelial sheets eventually break up and the cells continue to migrate individually (Figure 4.2.d), a process which some suggest also occurs *in vivo*[5 109].

The second accomplishment (successfully culturing trunk neural crest in Danilchik's medium) is also a break-through because previous experiments have found that while trunk neural crest explants grown in saline can attach to collagen-coated substrata, no cells emigrate from the explant[140]. However, the present study shows that, in Danilchik's medium, trunk neural crest cells extracted from embryos at stages 19-21 attach, migrate and even differentiate into neurons as observed in the majority of cultures. This

migration pattern is similar to that which occurs *in vivo* because the individual cells move radially away from the explant. This finding led to the idea of using Danilchik's medium for more direct comparison of cranial and trunk neural crest cells in culture because cranial neural crest explants do not attach and migrate in an enriched medium. However, the potency of melanocyte differentiation is limited to the enriched medium as shown previously in this laboratory[140].

The third accomplishment (testing the effects of a low concentration of alcohol on both trunk and cranial neural crest cells and establishing that they respond differently to alcohol) provides new information regarding the effects of a low alcohol level on neural crest cells *in vitro*. Many investigations have demonstrated that neural crest cells are sensitive to alcohol[21-23 108]. However, only the effects of high alcohol levels on neural crest cells have been well investigated, while the effects of low alcohol levels have been poorly studied (Section 2.6-2.15). The present *in vitro* experiment was set up to test the effects of an alcohol level of 0.05% (equivalent to one standard drink) on neural crest cells under several different growing conditions. It was decided to give the alcohol as a single dose at the time of explantation. The experiments were run on both cranial and trunk neural crest cells, and during this process it was found that this low alcohol level does affect both populations, but differently (for details see Section 5.2). This finding is a break-through in this field, since it shows that even small amounts of alcohol can pose a significant risk to the development of neural crest cells and also that the response depends on the axial level of the neural crest studied.

Finally, the fourth accomplishment (providing a quantitative method for analysis) is also significant because the results have been analyzed quantitatively. To examine the effects of low alcohol levels on neural crest cell behavior, this study analyzed the morphological and locomotory responses of the cells. In an attempt to make the analysis as simple as possible four parameters were selected: cell count, cell shape, distance traveled, and cell size. The analysis of these parameters provided numerical values which were used to show similarities and differences between the tested groups. To increase the reliability and reproducibility of the measurements, a specifically designed computer program, MetaMorph, was used. Some limitations result from this approach because all of the parameters had to be measured manually. For example, the recognition of discrete shapes is somewhat arbitrary, as is the selection of the 20 most displaced cells around the explant. However, the results from the measurement error test were encouraging because this test performed better than expected (measurement error ranged from 0.8% to 5.3% with one exception, see Results Section 4.1) making the subsequent analysis reliable and reproducible. Therefore, this analytical method can also be useful for other studies of neural crest cells in culture.

5.2 Limitations of the *in vitro* model

This *in vitro* model allows for better control and manipulation of both the cells and their environment, features that are essential in understanding the mechanisms underlying the developmental effects of alcohol. However, it is difficult to extrapolate the findings from a small number of cells in isolation to the complex interactions that occur in developing embryos. In addition, cranial neural crest cells have been grown only in saline, a condition that is simpler than in a developing embryo. Therefore, the results of these *in vitro* experiments must be interpreted with caution.

Also, the different measurement times for cranial and trunk neural crest cells were selected based on the cells' behaviors. Cranial neural crest explants attach and liberate individual cells at 3h, while for trunk neural crest this occurs at 12-24h. Cranial neural crest cells begin to round up at 5h, while for trunk neural crest this begins at 48h. Thus, although the measurement times are different, the behaviors are similar, making comparisons more valid. However, it must be noted that cranial neural cells were in the test solution for a much shorter period than trunk neural crest cells. This could influence the results. Also, alcohol concentrations were not measured at the different times.

There are additional ways in which to expand these experiments, among them: add the alcohol at similar behavior times; for example, at initial cell migration or liberation of single cells; or attempt to account for normal drinking behavior by adding the alcohol slowly and not all at once, or use avian or mouse as the animal model as they

are higher vertebrates and therefore closer to humans in evolutionary terms. All of these interesting modifications remain to be explored with this system.

5.3 Findings associated with neural crest cell exposure to low alcohol levels

The results demonstrate that low alcohol levels can affect *Xenopus* neural crest cells *in vitro*. However, the responses differ between cranial and trunk neural crest cell populations.

5.3.1 Adverse effects on cell counts

Cranial neural crest cells are sensitive to alcohol during the phase in which individual cells are leaving the epithelial sheets; their numbers are negatively affected. In the embryo this could result in fewer cells reaching their final destinations at the appropriate times, with the consequence of smaller differentiated elements. The lack of change in the individual trunk neural crest cell counts at either interval demonstrates that these cells are less sensitive, or insensitive to alcohol, at least under the present experimental conditions.

5.3.2 Altered cell shapes

With respect to cell shape, low alcohol levels do not affect trunk neural crest cells to nearly the same extent as cranial neural crest cells. For cranial neural crest cells, the number of elongated and round cells decreased significantly at both measured times. For trunk neural crest cells, only the number of round cells increased significantly at one time – 24h.

The less significant change in trunk neural crest cell shape compared to cranial neural crest is another significant difference in response between the two cell populations. In the embryo, changes in cell shape could result in abnormal cell arrangement and patterns either due to morphological changes of cell outline or altered morphological ability, for example, if cells change from a fibroblastic-like to a stellate morphology.

5.3.3 Interference with cell displacement

With respect to migration distances of individual cells, cranial neural crest cells do not migrate as far in the presence of alcohol compared to cranial controls. *In vivo*, this could also have serious consequences. If cells stop short of their final destination, not only could structures develop in abnormal locations, but also the cells may miss crucial signals controlling their further development.

Trunk neural crest displacement is not affected by alcohol except on fibronectin where alcohol-treated cells migrated further than controls. This will be discussed below under "Proposed mechanisms of alcohol-induced damage to neural crest cells". Although this could be interpreted as a "positive" result, it would be negative if it happened in the embryo. Cells that migrate past their appropriate locations are just as much in the wrong location as are cells that do not migrate far enough. This would also lead to defective tissue pattern formation.

5.3.4 Disruption in cell sizes

Alcohol also affects cranial neural crest cell size at 3h (Figure 4.3.4.1). The cells become significantly larger than 3h controls. *In vivo*, this could also lead to developmental anomalies, possibly enlarged tissue masses.

Trunk neural crest cultured under all growing conditions does not, however, show significant differences in cell size between the control and experimental groups at either interval (Figure 4.4.5.1). Thus, again, the effects of alcohol vary depending on the axial level of the cultures, with cranial neural crest more sensitive than trunk neural crest under these conditions.

5.4 Proposed mechanisms of alcohol-induced damage to neural crest cells

Identifying the molecular mechanisms contributing to alcohol-induced neural crest cell damage is complicated by many factors. The first problem is that scientists have not yet completely determined the cellular and molecular processes involved in guiding and regulating normal cranial neural crest development (see Sections 2.1-2.6). A second problem is that alcohol is known to react with neural crest cells in many ways: through free-radicals, alteration of cell cytoskeleton, regulation of gene expression and/or reduction of retinoic acid (described in detail in Sections 2.12-2.15). This multiplicity of responses makes it difficult to discern the initial insult to the cells as well as the following cascade of cellular responses. Furthermore, there is no doubt about the effects of high alcohol levels, corresponding to chronic alcohol use or binge drinking behavior: neural crest cell death is inevitably the endpoint of these interactions (as previously described). The effects of low alcohol levels, however, are not as striking and therefore more difficult to establish. Finally, as shown by the present work, cranial and trunk neural crest cells appear to respond differently to alcohol.

5.4.1 Different developmental fates

Cranial and trunk neural crest cells share some properties (both migrate from neuroepithelium and both give rise some similar derivatives, for example neurons) but they also have some significant differences. Cranial neural crest gives a rise to a wide

array of derivatives and overall cranial neural crest cells remain together, forming large structures, for example, the mandible[31 65].

Furthermore, in *Xenopus Laevis*, as noted previously, early migratory cranial neural crest cells are aggregated into large masses which spread down the sides of the head[109]. Within these masses, the cells are attached to one another by intercellular junctions (Milos, personal communication). The cells in the initial masses are not mingled with mesoderm but are on top of it. However, partway during their relocation there is a presumed change in adhesiveness and mingling with mesoderm results. This may be analogous to the liberation of single cells that is observed from epithelial sheets *in vivo* (Milos, personal communication).

Trunk neural crest cells are more restricted in their derivatives and they tend to migrate more widely and as individuals[15 16]. Also, they are more versatile with respect to their migration substrates[10 33 72 136 140].

Overall, under the present experimental conditions, cranial neural crest cells appear to be more sensitive to alcohol. This is also seen in vivo with the preferential manifestation of FASD in the head. What mechanisms could be altered in the cells by alcohol, and what could account for the differences between the two populations? The following section will offer some possibilities that could be further explored.

5.4.2 Cell death

As mentioned above high concentrations of alcohol have been correlated with cranial neural crest cell apoptosis[21] [108]. The present work raises the possibility that apoptosis occurs in cranial neural crest explants in low concentrations of alcohol as indicated by the low number of cells that migrated out of the cell sheets. Since trunk neural crest cell numbers are not affected it is possible that a particular subset of neural crest cells that is restricted to cranial explants (the skeletal precursors) is pushed into apoptic cell death by alcohol. Alternatively, a recovery phase might occur in trunk neural crest. This interesting possibility remains to be explored.

5.4.3 Differences in modulating cell-cell adhesion

The smaller number of individual cranial neural crest cells that migrate from the cell sheets in the presence of alcohol could also reflect a delay in achieving a full migratory morphology as stated above. As already mentioned, neural crest cells in early migratory groups are attached to one another by intercellular junctions (Milos, personal communication). Individual cells may be liberated once these junctions disappear. Perhaps alcohol interferes with this epithelial/ mesenchymal transformation.

Cell-cell adhesion molecules such as N-CAM (a member of the immunoglobulin superfamily) may also be of interest. These molecules, which link the cells together and

are found in both trunk and cranial neural folds, are gradually lost during cell migration [2] [17]. In the head, antibodies against N-CAM cause abnormalities in the cranial neural tube and neural crest [17] and experimental evidence also suggests that N-CAM may be sensitive to alcohol. For example, exposure of embryonic brain cells to high alcohol levels causes abnormal density of N-CAM on the surface of migrating neurons, resulting in inhibition of their migration [54].

If alcohol does interfere with N-CAM removal, then delayed dispersion should also be observed in trunk neural crest cell cultures (since N-CAM is present in both cell populations at the onset of migration as indicated above). However, it was not observed under any of the tested conditions. This suggests that trunk neural crest cells might be "less sensitive" or may be able to recover from the alcohol-induced effects.

5.4.4 Differences in compensatory capabilities in cell-substrate interactions – Possible involvement of integrins

While impairment of cell-cell interactions may be involved in ethanol-induced defects of neural crest cells, cell-substrate interactions may also be important. Many studies have made use of sensitive techniques to track the movement of neural crest cells *in vivo* (described in Sections 2.3); however, there are still many fundamental questions regarding the molecular mechanisms regulating cell movement.

With respect to cranial and trunk neural crest cell movement, numerous studies have shown that there are regional differences between the two cell populations. For example, in culture, trunk neural crest cells of many vertebrates attach and migrate successfully on fibronectin, laminin and collagen[10 33 72 136 140], while cranial neural crest cells of higher vertebrates (such as birds) attach and migrate on fibronectin and laminin but not on collagen[72]. Amphibian cranial neural crest cells attach and migrate only on fibronectin[5]. The cell surface receptors involved in these migrations are the integrins discussed above (Section 2.4).

Perris et al[97] demonstrated that trunk neural crest cells interact with collagen I through an $\alpha 1\beta 1$ integrin. Since cranial neural crest cells do not utilize the $\alpha 1$ subunit of integrins for attachment to extracellular matrices, they cannot attach to collagen[72].

Furthermore, even though the results have demonstrated that both cranial and trunk neural crest cells attach and migrate on fibronectin, their migratory behavior may not be identical, or even similar, when cultured on this substrate. Past investigations have shown that, on fibronectin, avian trunk neural crest cells express a great variety of integrins: $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha v\beta 1$, $\alpha v\beta 3$, and a $\beta 8$ species[131]. While the $\alpha v\beta 1$ and $\alpha 8\beta 1$ species are involved in cell adhesion, only the $\alpha 4\beta 1$, $\alpha v\beta 3$, $\alpha 8\beta 1$, and more indirectly $\alpha 3\beta 1$ species are involved in cell migration. Alpha$5\beta 1$ and $\beta 8$ integrins are not significant in either adhesion or migration[131].

In contrast, during their migration on fibronectin, cranial neural crest cells have been shown to express only two of the integrins implicated in trunk neural crest cell migration, α5β1 and α3β1[5]. Interestingly, blocking of the α5β1 integrin of *Xenopus* cranial neural crest has demonstrated that interfering with this adhesion molecule only can completely stop cranial neural crest cell migration[5]. This suggests that normal functioning of this molecule is obligatory.

This further raises a question. In the trunk, does the wide range of integrins mean that the cells are "tougher"? If one species of integrin is interfered with (for example by alcohol) can other integrins compensate? In fact, trunk neural crest cell migration is significantly decreased only when both β1 and β3 families are blocked in combination, as opposed to individually, suggesting that compensation can occur: if β1 is blocked, then β3 family takes over, and vice-versa [131].

No studies have been done to investigate if alcohol affects integrin function. An interesting speculation is that, on fibronectin, alcohol affects the β1 family of integrins. Since cranial neural crest only uses only α5β1 integrins [5] this affects their migration. However, since compensation can occur with trunk neural crest, migration can still occur because the β3 family is still active. Depending on the migration-promoting characteristics of the β3 family, this might result in enhanced trunk neural crest cell displacement in the presence of alcohol. This speculation is a subject for future experimentation.

The observations also demonstrate that alcohol does not have a positive impact on trunk neural crest cell displacement on collagen, as it does on fibronectin. There is however a significant difference in the number of integrins expressed by trunk neural crest cells on these two substrates. On fibronectin, neural crest cells express seven integrins [131], while on collagen neural crest cells express only one (totally different) integrin, $\alpha 1\beta 1$ ([97] and unpublished results from Testaz laboratory as indicated in Testaz et al, 1999[131]). This could explain not only why trunk neural crest cells grown on fibronectin behave differently from those grown on collagen but also why they respond differently to alcohol.

Therefore, although this study did not test the effects of low alcohol levels on integrins, the results suggest that these levels of alcohol do affect cell-matrix interactions perhaps through various integrins.

5.5 Conclusion

The principal finding of this study is that although both cranial and trunk neural crest of frogs can survive and migrate in an alcohol level equivalent to one standard drink over a wide range of time (behavior that is significantly different from when the cells are exposed to high alcohol levels), their behavior is significantly altered.

A significant number of cranial neural crest cells leave the explant in the presence of a low alcohol level, but their displacement distance is significantly reduced. Their

migration as individual cells is delayed, and morphological changes are also observed. On the other hand, trunk neural crest cells appear to be "less sensitive" to a low alcohol level. A most interesting finding of this study is that on fibronectin trunk neural crest cells actually migrate farther following ethanol exposure than they do in the control group. On collagen, however, experimental trunk neural crest cells do not differ significantly from controls.

Although full FASD has been shown to be caused by fetal exposure to high alcohol levels, this study has demonstrated that neural crest cells may also be affected by low alcohol levels, with cranial neural crest cells being more vulnerable than trunk neural crest cells. Therefore even low alcohol levels could be detrimental to normal craniofacial development.

Can the present findings, however, be extrapolated to humans? Certainly lower vertebrates such as amphibians are farther away from humans than birds or mammals in evolutionary terms. However, both amphibians and humans have neural crest cells which contribute to the facial skeleton. Because the aim of this study was to evaluate the basic responses of neural crest cells to a low alcohol level it was appropriate to study them in a simple organism such as the frog.

The insights gained from this study provides numerous opportunities for researchers to use various animal models combined with the new technologies of cellular, molecular and developmental biology to explore the possible underlying mechanisms

associated with fetal exposure to low alcohol levels. This work should accelerate the development of rational approaches to the diagnosis, treatment, and prevention of alcohol-induced birth defects and provide more insight into the development of FASD.

VI

CHAPTER SIX

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APPENDIX A

RECIPES/PROCEDURES

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RECIPIES

Danilchik's medium (200ml)

NaCl	53mM (0.625g)
Na_2CO_3	10mM (0.212g)
K-Gluc	4.25mM (0.199g)
$MgSO_4$	1mM (0.024g)
$CaCl_2 \cdot 2H_2O$	1mM (0.029g)
Bicine	20mM (0.653g)
Gentamicin	10mg
dH_2O	200ml

adjust pH to 7.4

Niv-Twitty Solution (NTS) (1000ml)

Solution A

NaCl	3.4mg
KCl	50mg
$Ca(NO_3) \cdot 4H_2O$	80mg
$MgSO_4$	100mg
dH_2O	500ml

Solution B

Na_2HPO_4	110mg
KH_2PO_4	20mg
dH_2O	250ml

Solution C

$NaHCO_3$	200mg
dH_2O	250ml

Mix all 3 solutions, adjust pH to 7.4

Agar dishes (12 dishes)

Agar	2g
dH_2O	100ml

APPENDIX B

DOSE CALCULATION

Dose Calculation

Alcohol concentrations have been calculated based on the amount of pure alcohol in 45 ml of spirit (40% pure ethanol per volume) that would be dissolved in the total volume of fluid in the body (≈ 45L). The calculations are as follow:

One standard drinks

P% - percentage by volume

$V_1 = 45L = 45000$ ml – volume of body fluid in an average weight human body

$V_2 = 45$ ml – volume of one standard drink with k% = 40%

X = volume of pure alcohol in one standard drink

$V = V_1 + V_2 = 45045$ ml - volume of total body fluid after ingestion of one standard drink

$X = (k\%/100\%)*V_2 = (40\%/100\%)* 45ml = 18ml$

$P\% = [X / V]* 100\% = [18/45045 ml)* 100\%] \approx 0.04\% \approx 7mM$

One beer

P% - percentage by volume

$V_1 = 45L = 45000$ ml – volume of body fluid in an average weight human body

$V_2 = 350ml$ – volume of one beer with k% = 5%

X = volume of pure alcohol in one beer

$V = V_1 + V_2 = 46065$ ml - volume of total body fluid after ingestion of one beer

$X = (k\%/100\%)*V_2 = (5\%/100\%)* 350ml = 17.5ml$

$P\% = [X / V]* 100\% = [17.5/45350 ml)* 100\%] \approx 0.04\% \approx 7mM$

BAC between 0.4 % -0.6% are usually fatal due to organ failure (ADDAC)

100mM = 0.1M what is the concentration of ETOH?

n = m/M
0.10M = m/ 46
m = 4.6 g ⟶ V = m/d = 5.76ml

5.76ml –1000 ml
X - 100 ml
X = 0.576 ml ⟶ 0.576ml in 100 ml = 0.576 % ⟶ 15 standard drinks

APPENDIX C

STATISTICAL ANALYSIS

Legend for Statistical Analysis (SPSS format)

Group Statistic Table:
Summarizes statistics for all of the groups (group size, mean, standard deviation, standard error mean).

Normal Q-Q Graph:
Tests whether the shape of a distribution of the entire data N=30 (○ –individual data score) is normal. If the data points fall on the straight line the distribution is normal. Departure from the straight-line suggests a nonnormal population distribution. Q-Q plots compare the obtained scores on the x-axis with the expected Z score from the normal probability table grafted on the y-axis.
[Fewer data points indicate ties or fewer cultures available for the measurements.]

Boxplot:
Plots the smallest observation, lower quartile (from the lowest measurement to the 25th percentile), median (average of the middle observations in the order list), upper quartile (from the 75th percentile to the largest measurement), largest observation, and outlying or extreme values.

1. Variability:

The height of the box represents the difference between the 25^{th} and 75^{th} percentile. The height of the box determines the variability. The larger the box, the greater the spread of the data.

2. Central Tendency:

The horizontal line inside the box represents the median (a point below which fifty percent of the cases fall). If the median is not in the centre of the box, the distribution may be skewed. If the median line is closer to the lower edge of the boxplot, this suggests a concentration of the values in the lower part of the middle half. If the median line is closer to the upper edge of the box, this suggests a concentration of the values in the upper part of the half.

3. Whiskers:

Whiskers: Draw lines from the ends of the box to the largest and the smallest values that are not outliers. If whiskers are of different length, the data distribution may be skewed. A longer upper whisker suggests that the data is positively skewed, while a longer lower whisker suggests that data is negatively skewed.

4. Outliers:

Case numbers are used to label outliers (○) and extremes (*). The boxplot shows detected outliers and extremes. The outliers are cases with values between 1.5 and 3 box lengths from the 75th percentile or 25th percentile. The extremes are values more than 3 box-lengths from the 75th or 25th percentile.

Each boxplot was also tested statistically for variability and skewedness using Levene's Test for Equality of Variance and Kolmogorov-Smirnov Test for Normality respectively. $p<0.05$ was considered statistically significant.

Statistical Analysis:

The visualization of the boxplots and the Levene's test for Equality of Variances indicate major problems with the homogeneity of variances in this data. The visualization of the boxplots and the Kolmogorov-Smirnov Test for Normality also revealed different degrees of skewedness. Because I argued that data transformation could obscure the factors making the distribution skewed to begin with [especially when considering the sample size], I selected the Wilcoxon Rank of Sum test for independent data (Mann-Whitney U test). The Wilcoxon Rank of Sum test is a non-parametric analog of the independent t-test, which is based on ranks.

The Mann-Whitney U ranks all of the cases from the lowest to the highest score. The "Mean Rank" is the mean of those ranks for each group and the "Sum of Ranks" is the sum of those ranks for each group (shown in the Ranks Tables).

U (shown in the Test Statistics table as Mann-Whitney U) is defined as the smaller of the two compared U's (U_1 and U_2; see formula below). If U is significant there is a difference between the two compared groups. R (shown in the Test Statistics table as Wilcoxon W) defines the smaller of the two compared R's, aka. the Sum of Ranks (R_1 or R_2). R_1 or R_2 are used to calculate U_1 and U_2 respectively.

$$U_1 = n_1 n_2 + (n_1 (n_1+1))/2 - R_1$$

$$U_2 = n_1 n_2 + (n_2 (n_2+1))/2 - R_2$$

where

n_1 = number of observations in group 1
n_2 = number of observations in group 2
R_1 = sum of ranks assigned to group 1
R_2 = sum of ranks assigned to group 2

When the sample size is large, the sampling distribution of U approaches a normal curve and the Z score can be reported. The calculated p-value is obtained from the Z score. Because the sample sizes for both groups are 15, the Asymptotic significance [Asymp. Sig. (2-tailed)], rather than the Exact significance (2*(1-tailed Sig.), was chosen for reporting a significant difference.

Note:
ETOH = ethanol (alcohol)

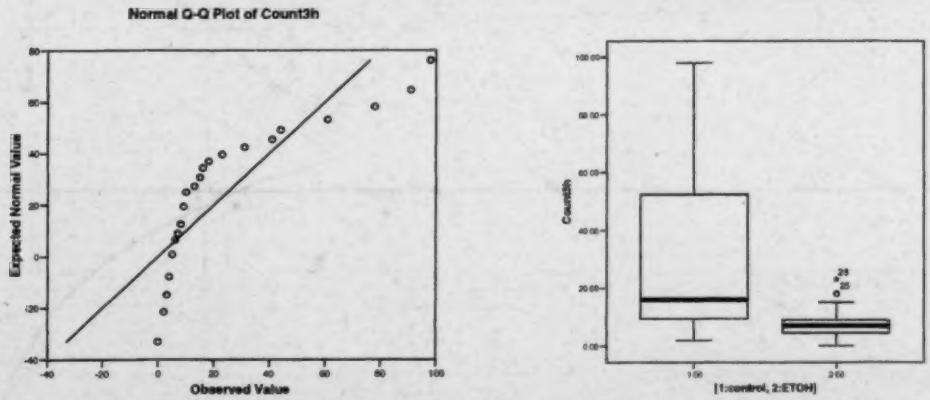
Page

1.1 Cranial neural crest cells / Fibronectin / Danilchik's medium / Count

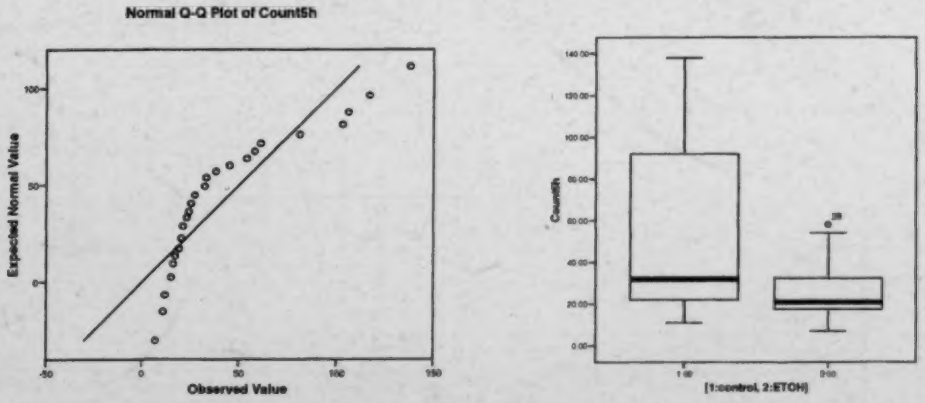
1.1.1 Comparison between control and alcohol-treated cranial neural crest cells

Group Statistics

	[1:control, 2:ETOH]	N	Mean	Std. Deviation	Std. Error Mean
Count3h	1.00	15	34.8000	32.76148	8.45898
	2.00	15	8.3333	6.09059	1.57258
Count5h	1.00	15	54.6000	43.15222	11.14186
	2.00	15	26.4667	14.36199	3.70825



Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)



Markedly skewed data in the control group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	[1:control, 2:ETOH]	N	Mean Rank	Sum of Ranks
Count3h	1.00	15	19.97	299.50
	2.00	15	11.03	165.50
	Total	30		
Count5h	1.00	15	18.30	274.50
	2.00	15	12.70	190.50
	Total	30		

Test Statistics[b]

	Count3h	Count5h
Mann-Whitney U	45.500	70.500
Wilcoxon W	165.500	190.500
Z	-2.784	-1.743
Asymp. Sig. (2-tailed)	.005	.081
Exact Sig. [2*(1-tailed Sig.)]	.004[a]	.081[a]

a. Not corrected for ties.

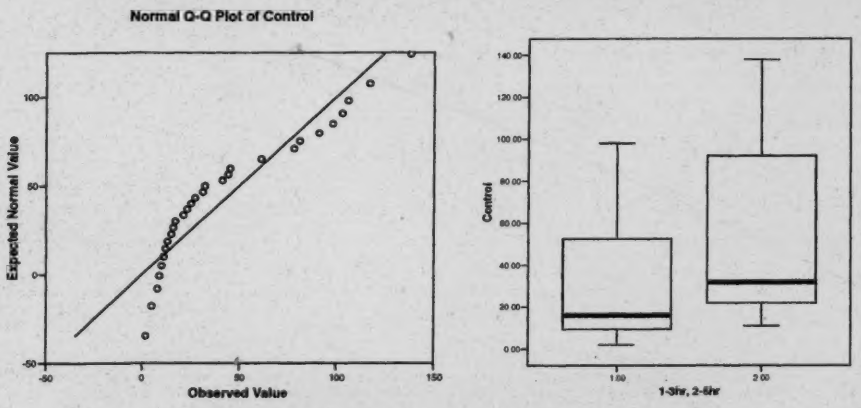
b. Grouping Variable: [1:control, 2:ETOH]

Page

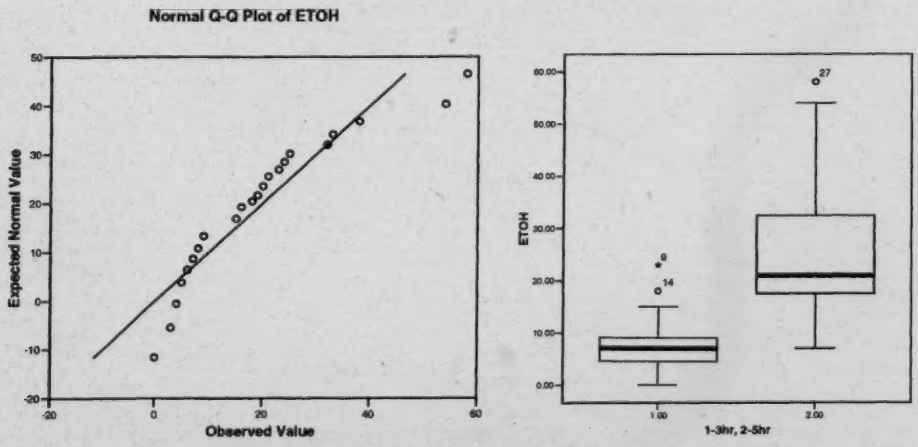
1.1.2 Comparison of control and alcohol-treated cranial neural crest cells between intervals

Group Statistics

	1-3hr, 2-5hr	N	Mean	Std. Deviation	Std. Error Mean
Control	1.00	15	34.8000	32.76148	8.45898
	2.00	15	54.6000	43.15222	11.14186
ETOH	1.00	15	8.3333	6.09059	1.57258
	2.00	15	26.4667	14.36199	3.70825



Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)



Markedly skewed data in the 3h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-3hr, 2-5hr	N	Mean Rank	Sum of Ranks
Control	1.00	15	12.70	190.50
	2.00	15	18.30	274.50
	Total	30		
ETOH	1.00	15	9.23	138.50
	2.00	15	21.77	326.50
	Total	30		

Test Statistics[b]

	Control	ETOH
Mann-Whitney U	70.500	18.500
Wilcoxon W	190.500	138.500
Z	-1.742	-3.904
Asymp. Sig. (2-tailed)	.081	.000
Exact Sig. [2*(1-tailed Sig.)]	.081[a]	.000[a]

a. Not corrected for ties.

b. Grouping Variable: 1-3hr, 2-5hr

Page

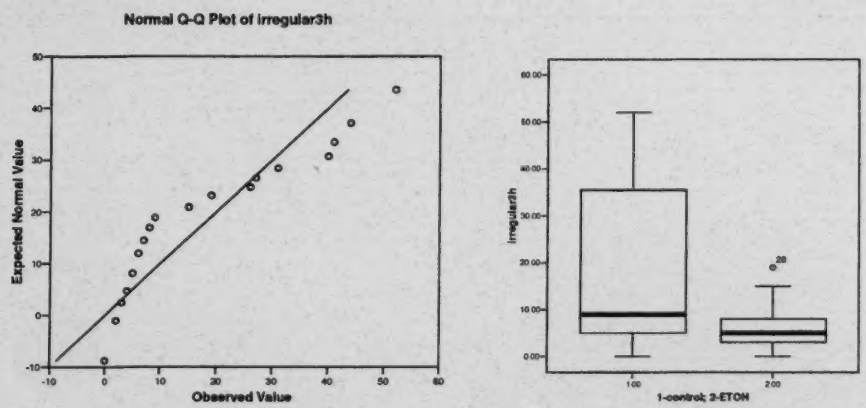
1.2 Cranial neural crest cells / Fibronectin / Danilchik's medium / Shape

1.2.1 Comparison between control and alcohol-treated cranial neural crest cells

Group Statistics

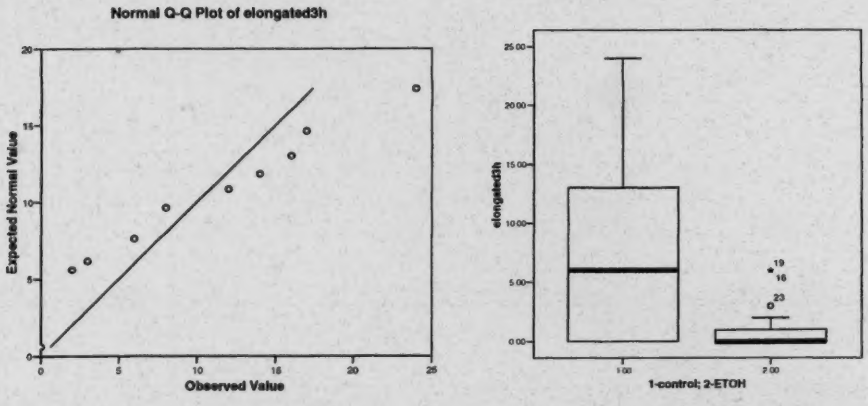
	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
irregular3h	1.00	15	19.8000	18.18634	4.69569
	2.00	15	6.5333	5.78010	1.49241
elongated3h	1.00	15	7.4000	7.74412	1.99952
	2.00	15	1.1333	2.16685	.55948
round3h	1.00	15	7.6000	15.08452	3.89481
	2.00	15	.6667	1.39728	.36078
irregular5h	1.00	15	34.6667	32.17956	8.30873
	2.00	15	20.2000	15.03425	3.88183
elongated5h	1.00	15	9.9333	7.20582	1.86053
	2.00	15	3.4667	4.45400	1.15002
round5h	1.00	15	10.0000	14.26785	3.68394
	2.00	15	2.8000	6.22438	1.60713

3h interval

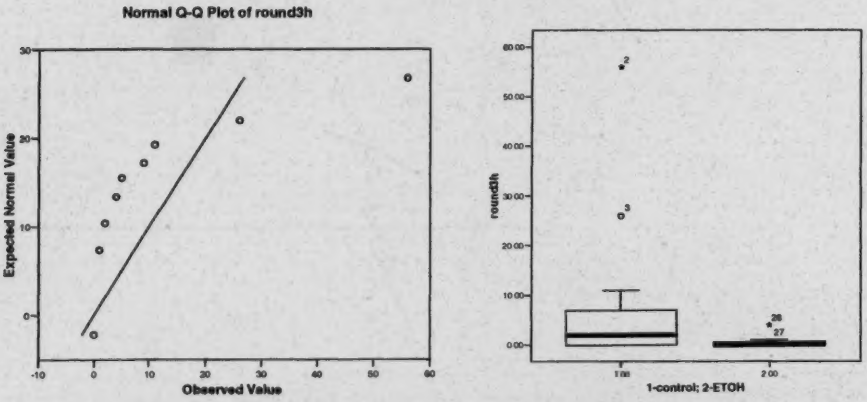


Markedly skewed data in the control group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

Page



Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$),, unequal variance (Levene's Test for Equality of Variance $p<0.05$)



Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

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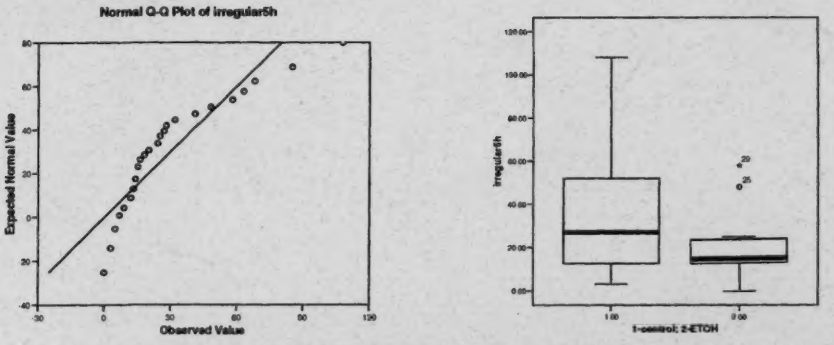
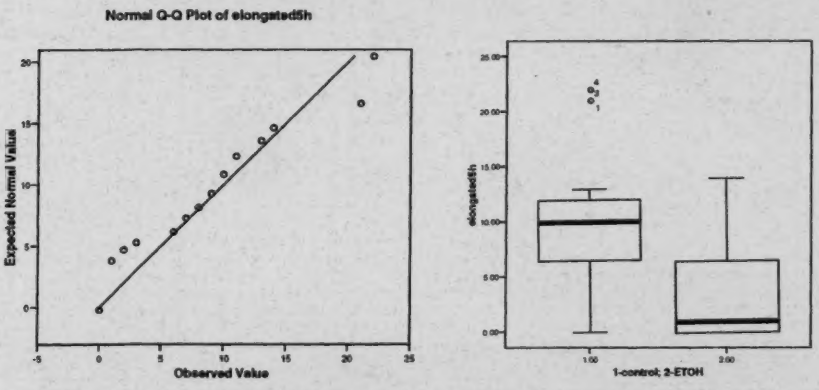
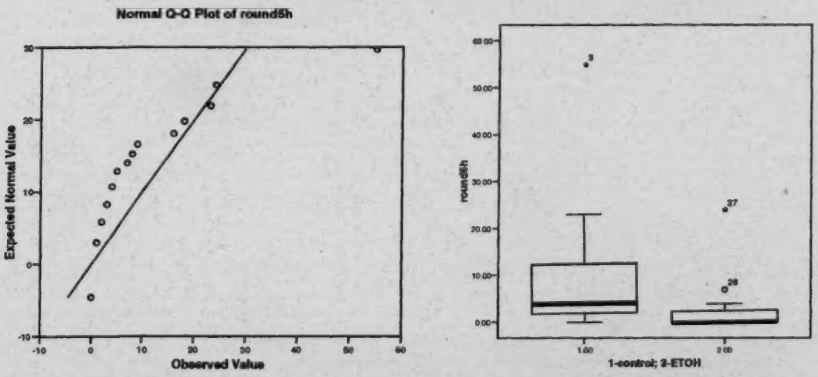
5h interval



Markedly skewed data in ETOH group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)



Markedly skewed data in ETOH group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)



Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
irregular3h	1.00	15	18.43	276.50
	2.00	15	12.57	188.50
	Total	30		
elongated3h	1.00	15	19.07	286.00
	2.00	15	11.93	179.00
	Total	30		
round3h	1.00	15	18.60	279.00
	2.00	15	12.40	186.00
	Total	30		
irregular5h	1.00	15	17.03	255.50
	2.00	15	13.97	209.50
	Total	30		
elongated5h	1.00	15	19.40	291.00
	2.00	15	11.60	174.00
	Total	30		
round5h	1.00	15	19.53	293.00
	2.00	15	11.47	172.00
	Total	30		

Test Statistics[b]

	irregular3h	elongated3h	round3h	irregular5h	elongated5h	round5h
Mann-Whitney U	68.500	59.000	66.000	89.500	54.000	52.000
Wilcoxon W	188.500	179.000	186.000	209.500	174.000	172.000
Z	-1.830	-2.457	-2.135	-.955	-2.462	-2.561
Asymp. Sig. (2-tailed)	.067	.014	.033	.340	.014	.010
Exact Sig. [2*(1-tailed Sig.)]	.067[a]	.026[a]	.056[a]	.345[a]	.015[a]	.011[a]

a. Not corrected for ties.

b. Grouping Variable: 1-control; 2-ETOH

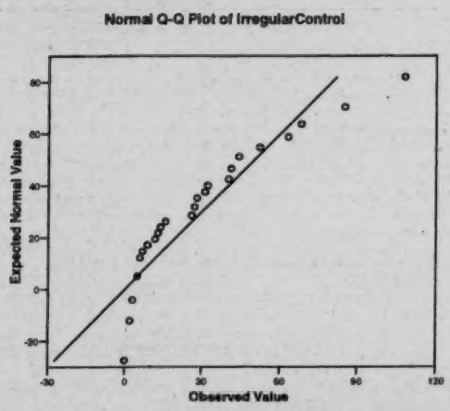
Page

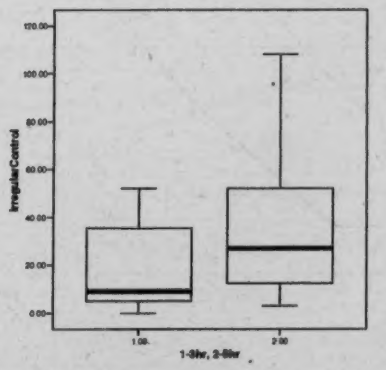
1.2.2 Comparison of control and alcohol-treated cranial neural crest cells between intervals

Group Statistics

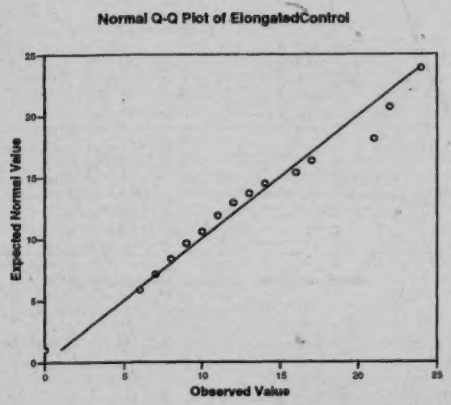
	1-3hr, 2-5hr	N	Mean	Std. Deviation	Std. Error Mean
IrregularControl	1.00	15	19.8000	18.18634	4.69569
	2.00	15	34.6667	32.17956	8.30873
ElongatedControl	1.00	15	7.4000	7.74412	1.99952
	2.00	15	9.9333	7.20582	1.86053
RoundControl	1.00	15	7.6000	15.08452	3.89481
	2.00	15	10.0000	14.26785	3.68394
IrregularETOH	1.00	15	6.5333	5.78010	1.49241
	2.00	15	20.2000	15.03425	3.88183
ElongatedETOH	1.00	15	1.1333	2.16685	.55948
	2.00	15	3.4667	4.45400	1.15002
RoundETOH	1.00	15	.6667	1.39728	.36078
	2.00	15	2.8000	6.22438	1.60713

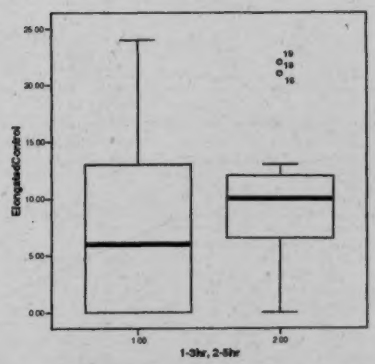
Control group



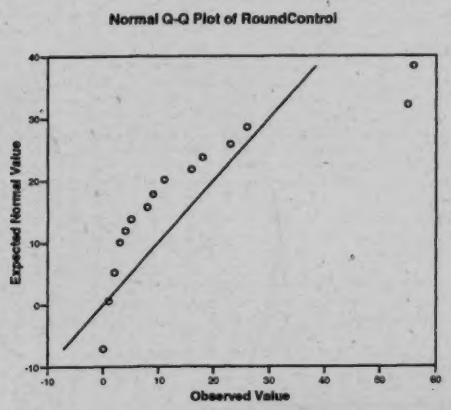


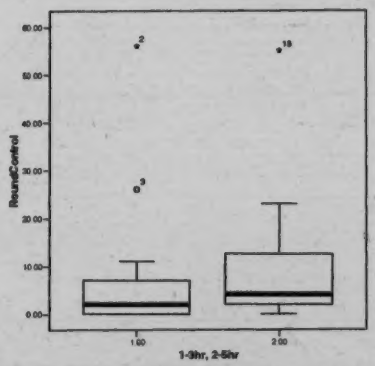
Markedly skewed data in the 3h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)





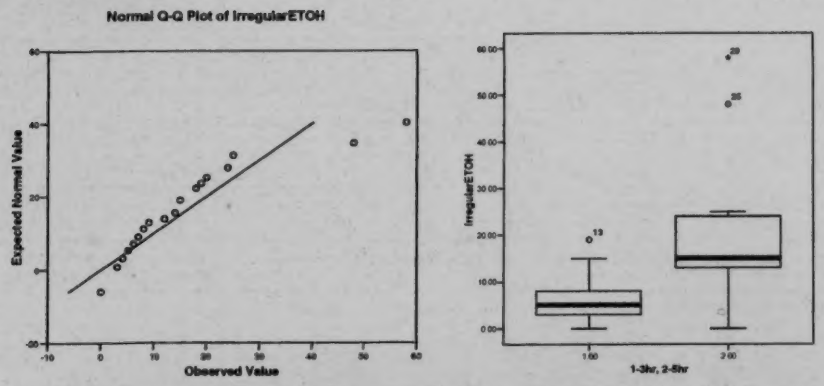
Markedly skewed data in the 3h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)



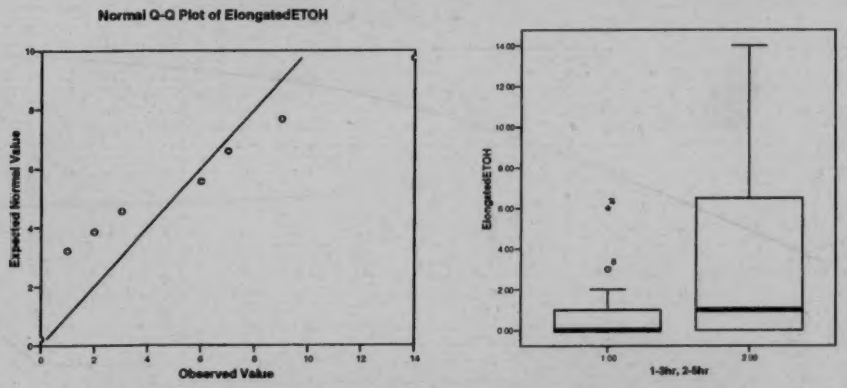


Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

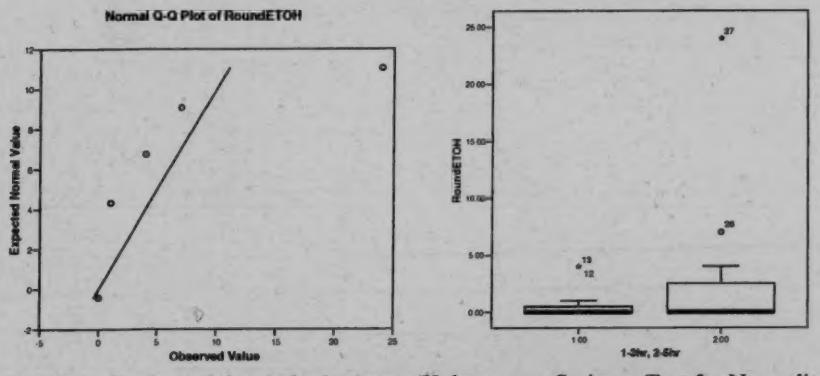
Page
ETOH group



Markedly skewed data in the 5h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)



Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)



Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-3hr, 2-5hr	N	Mean Rank	Sum of Ranks
IrregularControl	1.00	15	13.27	199.00
	2.00	15	17.73	266.00
	Total	30		
ElongatedControl	1.00	15	13.93	209.00
	2.00	15	17.07	256.00
	Total	30		
RoundControl	1.00	15	13.17	197.50
	2.00	15	17.83	267.50
	Total	30		
IrregularETOH	1.00	15	10.40	156.00
	2.00	15	20.60	309.00
	Total	30		
ElongatedETOH	1.00	15	12.80	192.00
	2.00	15	18.20	273.00
	Total	30		
RoundETOH	1.00	15	13.80	207.00
	2.00	15	17.20	258.00
	Total	30		

Test Statistics[b]

	Irregular Control	Elongated Control	RoundControl	IrregularETOH	Elongated ETOH	RoundETOH
Mann-Whitney U	79.000	89.000	77.500	36.000	72.000	87.000
Wilcoxon W	199.000	209.000	197.500	156.000	192.000	207.000
Z	-1.392	-.989	-1.472	-3.182	-1.859	-1.230
Asymp. Sig. (2-tailed)	.164	.323	.141	.001	.063	.219
Exact Sig. [2*(1-tailed Sig.)]	.174[a]	.345[a]	.148[a]	.001[a]	.098[a]	.305[a]

a. Not corrected for ties.

b. Grouping Variable: 1-3hr, 2-5hr

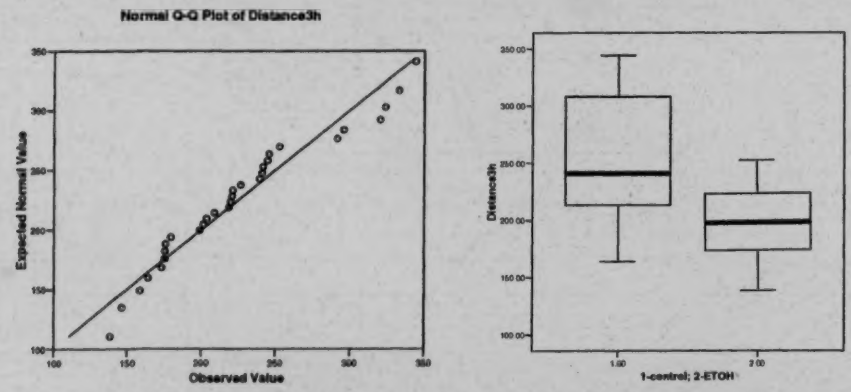
Page

1.3 Cranial neural crest cells / Fibronectin / Danilchik's medium / Distance

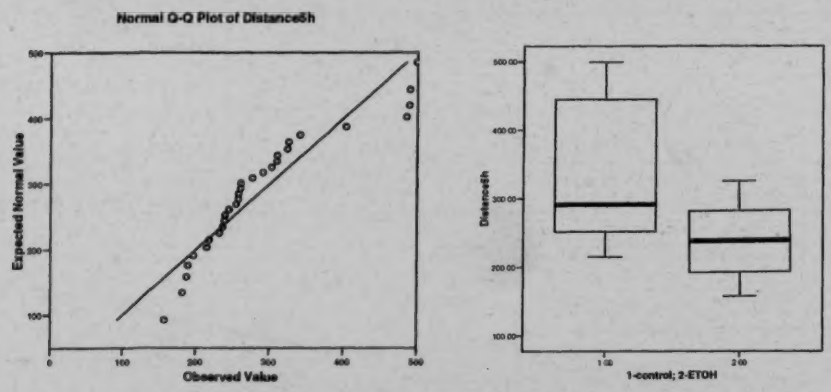
1.3.1 Comparison between control and alcohol-treated cranial neural crest cells

Group Statistics

	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Distance3h	1.00	15	254.8362	58.74029	15.16668
	2.00	15	197.0105	36.82149	9.50727
Distance5h	1.00	15	336.1305	107.13582	27.66235
	2.00	15	242.6502	54.82156	14.15487



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Distance3h	1.00	15	19.47	292.00
	2.00	15	11.53	173.00
	Total	30		
Distance5h	1.00	15	19.47	292.00
	2.00	15	11.53	173.00
	Total	30		

Test Statistics[b]

	Distance3h	Distance5h
Mann-Whitney U	53.000	53.000
Wilcoxon W	173.000	173.000
Z	-2.468	-2.468
Asymp. Sig. (2-tailed)	.014	.014
Exact Sig. [2*(1-tailed Sig.)]	.013[a]	.013[a]

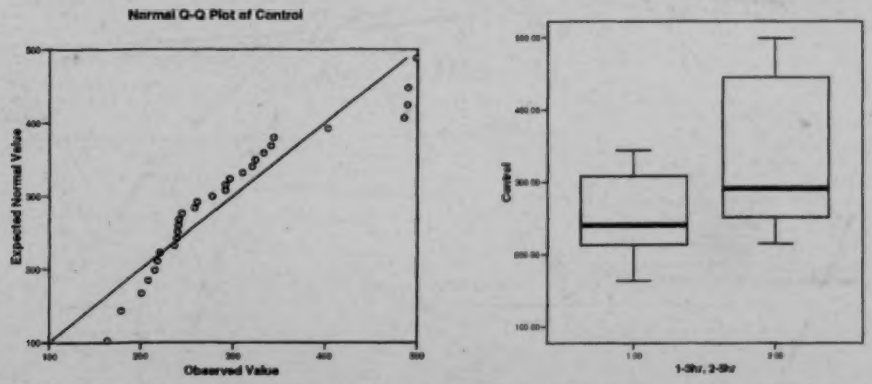
a. Not corrected for ties.

b. Grouping Variable: 1-control; 2-ETOH

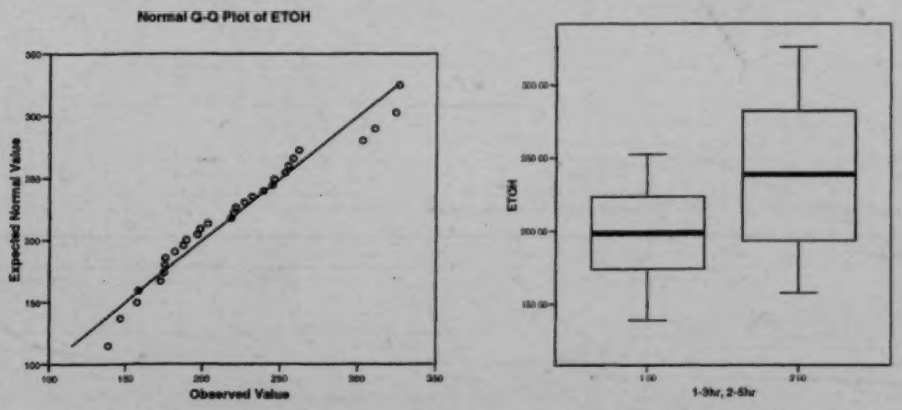
1.3.2 Comparison of control and alcohol-treated cranial neural crest cells between intervals

Group Statistics

	1-3hr, 2-5hr	N	Mean	Std. Deviation	Std. Error Mean
Control	1.00	15	254.8362	58.74029	15.16668
	2.00	15	336.1305	107.13582	27.66235
ETOH	1.00	15	197.0105	36.82149	9.50727
	2.00	15	242.6502	54.82156	14.15487



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-3hr, 2-5hr	N	Mean Rank	Sum of Ranks
Control	1.00	15	12.20	183.00
	2.00	15	18.80	282.00
	Total	30		
ETOH	1.00	15	11.80	177.00
	2.00	15	19.20	288.00
	Total	30		

Test Statistics[b]

	Control	ETOH
Mann-Whitney U	63.000	57.000
Wilcoxon W	183.000	177.000
Z	-2.053	-2.302
Asymp. Sig. (2-tailed)	.040	.021
Exact Sig. [2*(1-tailed Sig.)]	.041[a]	.021[a]

a. Not corrected for ties.

b. Grouping Variable: 1-3hr, 2-5hr

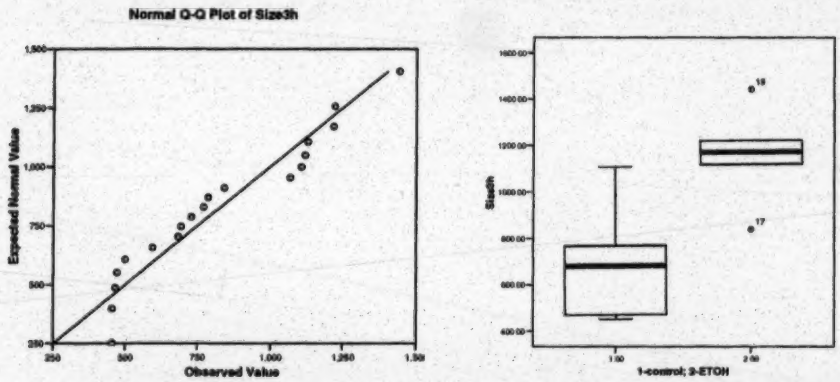
Page

1.4 Cranial neural crest cells / Fibronectin / Danilchik's medium / Size

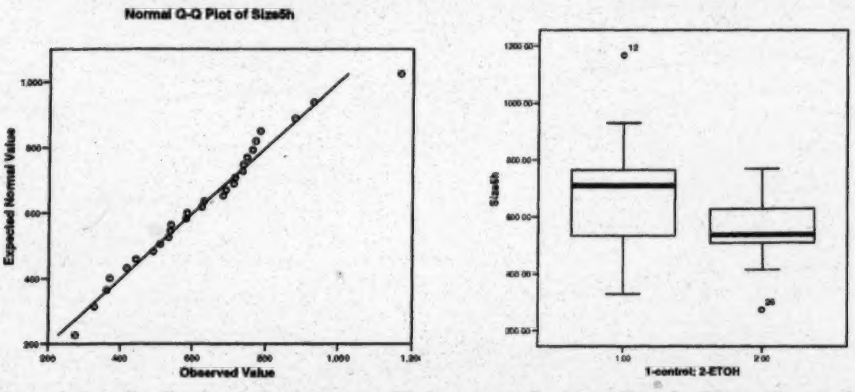
1.4.1 Comparison between control and alcohol-treated cranial neural crest cells

Group Statistics

	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Size3h	1.00	13	674.3137	221.18029	61.34437
	2.00	6	1161.7973	196.09752	80.05648
Size5h	1.00	15	677.3621	230.18692	59.43401
	2.00	13	566.6580	139.09338	38.57756



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05) note: small sample size in the ETOH group



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)

Page

NonParametric Test

\

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Size3h	1.00	13	7.15	93.00
	2.00	6	16.17	97.00
	Total	19		
Size5h	1.00	15	16.53	248.00
	2.00	13	12.15	158.00
	Total	28		

Test Statistics[b]

	Size3h	Size5h
Mann-Whitney U	2.000	67.000
Wilcoxon W	93.000	158.000
Z	-3.245	-1.405
Asymp. Sig. (2-tailed)	.001	.160
Exact Sig. [2*(1-tailed Sig.)]	.000[a]	.170[a]

a. Not corrected for ties.

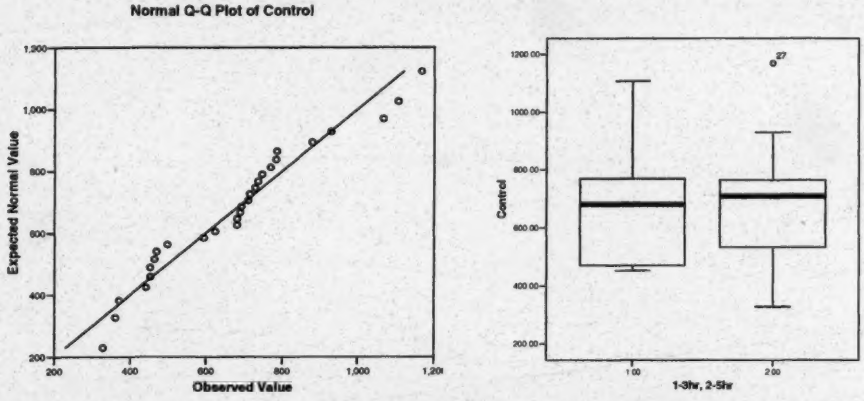
b. Grouping Variable: 1-control; 2-ETOH

Page

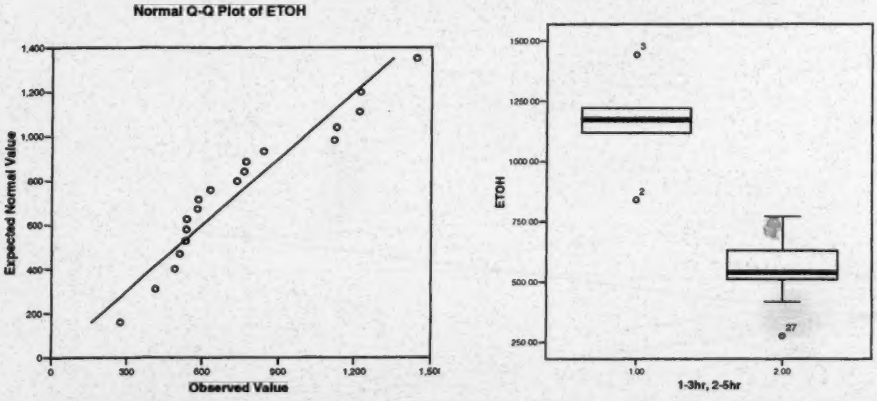
1.4.2 Comparison of control and alcohol-treated cranial neural crest cells between intervals

Group Statistics

	1-3hr, 2-5hr	N	Mean	Std. Deviation	Std. Error Mean
Control	1.00	13	674.3137	221.18029	61.34437
	2.00	15	677.3621	230.18692	59.43401
ETOH	1.00	6	1161.7973	196.09752	80.05648
	2.00	13	566.6580	139.09338	38.57756



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05). Note: small sample size in the ETOH group at 3h

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-3hr, 2-5hr	N	Mean Rank	Sum of Ranks
Control	1.00	13	14.46	188.00
	2.00	15	14.53	218.00
	Total	28		
ETOH	1.00	6	16.50	99.00
	2.00	13	7.00	91.00
	Total	19		

Test Statistics[b]

	Control	ETOH
Mann-Whitney U	97.000	.000
Wilcoxon W	188.000	91.000
Z	-.023	-3.421
Asymp. Sig. (2-tailed)	.982	.001
Exact Sig. [2*(1-tailed Sig.)]	1.000[a]	.000[a]

a. Not corrected for ties.

b. Grouping Variable: 1-3hr, 2-5hr

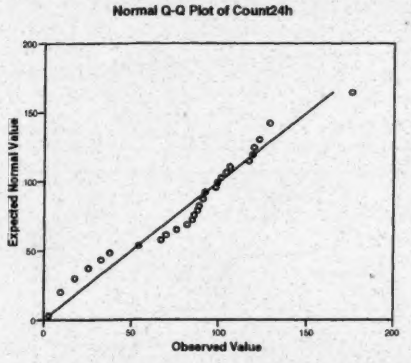
Page

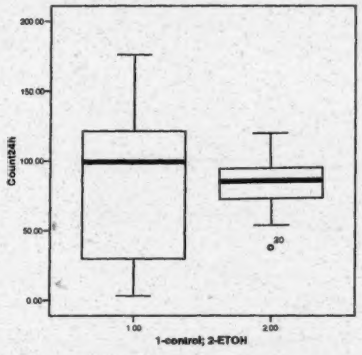
2.1 Trunk neural crest cells / Fibronectin / Danilchik's medium / Count

2.1.1 Comparison between control and alcohol-treated trunk neural crest cells

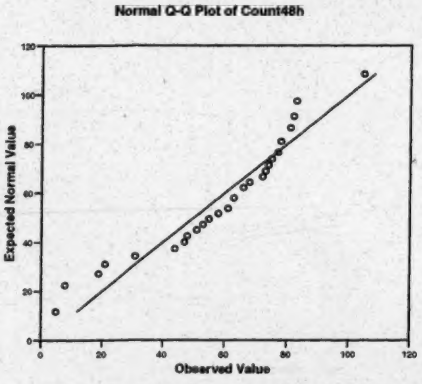
Group Statistics

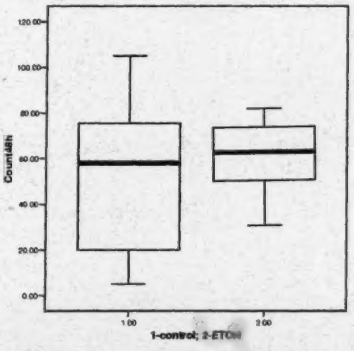
	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Count24h	1.00	15	83.4000	52.55990	13.57091
	2.00	15	84.6000	21.85602	5.64320
Count48h	1.00	15	51.9333	32.36062	8.35548
	2.00	15	61.8667	14.91340	3.85062





Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)





Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Count24h	1.00	15	16.57	248.50
	2.00	15	14.43	216.50
	Total	30		
Count48h	1.00	15	14.63	219.50
	2.00	15	16.37	245.50
	Total	30		

Test Statistics[b]

	Count24h	Count48h
Mann-Whitney U	96.500	99.500
Wilcoxon W	216.500	219.500
Z	-.664	-.540
Asymp. Sig. (2-tailed)	.507	.589
Exact Sig. [2*(1-tailed Sig.)]	.512[a]	.595[a]

a. Not corrected for ties.

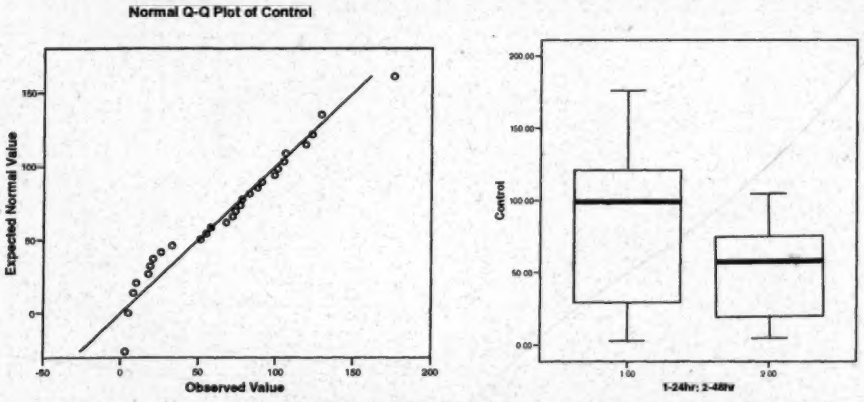
b. Grouping Variable: 1-control; 2-ETOH

Page

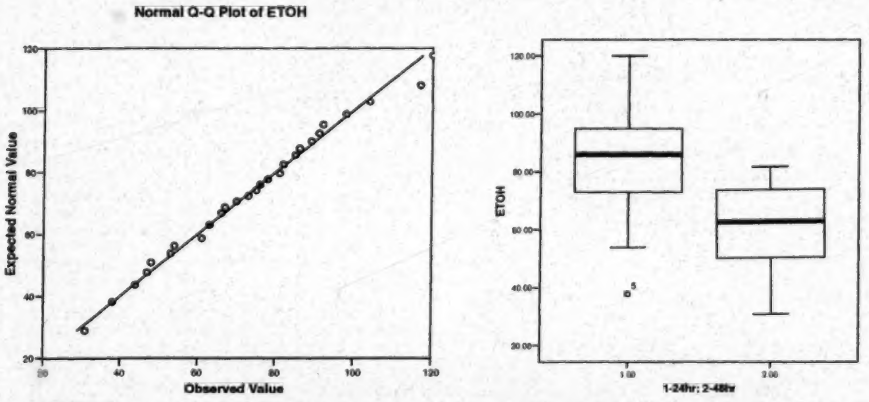
2.1.2 *Comparison of control and alcohol-treated trunk neural crest cells between intervals*

Group Statistics

	1-24hr; 2-48hr	N	Mean	Std. Deviation	Std. Error Mean
Control	1.00	15	83.4000	52.55990	13.57091
	2.00	15	51.9333	32.36062	8.35548
ETOH	1.00	15	84.6000	21.85602	5.64320
	2.00	15	61.8667	14.91340	3.85062



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-24hr; 2-48hr	N	Mean Rank	Sum of Ranks
Control	1.00	15	18.80	282.00
	2.00	15	12.20	183.00
	Total	30		
ETOH	1.00	15	20.50	307.50
	2.00	15	10.50	157.50
	Total	30		

Test Statistics[b]

	Control	ETOH
Mann-Whitney U	63.000	37.500
Wilcoxon W	183.000	157.500
Z	-2.054	-3.113
Asymp. Sig. (2-tailed)	.040	.002
Exact Sig. [2*(1-tailed Sig.)]	.041[a]	.001[a]

a. Not corrected for ties.

b. Grouping Variable: 1-24hr; 2-48hr

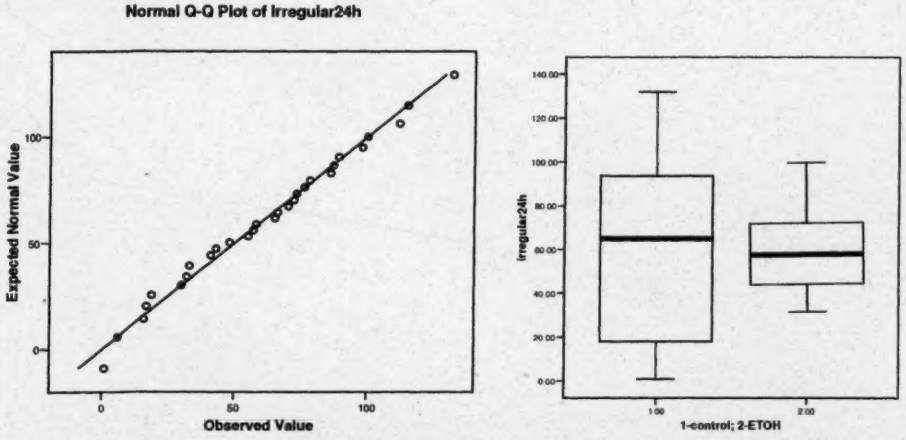
2.2 Trunk neural crest cells / Fibronectin / Danilchik's medium / Shape

2.2.1 Comparison between control and alcohol-treated trunk neural crest cells

Group Statistics

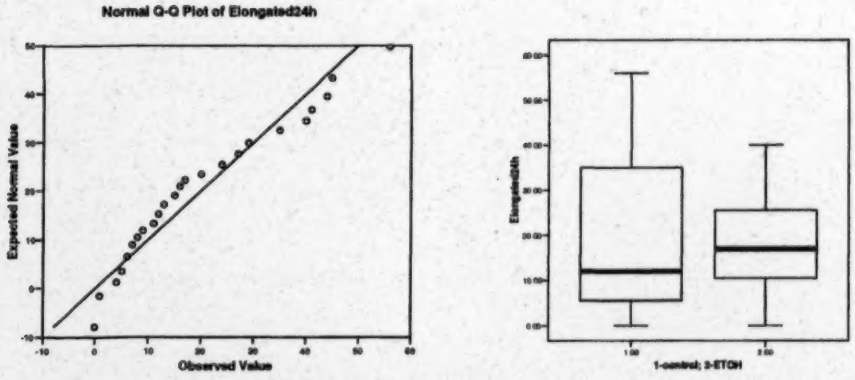
	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Irregular24h	1.00	15	61.0667	44.45136	11.47729
	2.00	15	59.4667	19.65730	5.07549
Elongated24h	1.00	15	19.2667	18.58750	4.79927
	2.00	15	18.8000	11.14963	2.87882
Round24h	1.00	15	3.0667	3.15021	.81338
	2.00	15	6.3333	4.09994	1.05860
Irregular48h	1.00	15	10.1333	11.88557	3.06884
	2.00	15	14.2667	5.72547	1.47831
Elongated48h	1.00	15	16.3333	16.09200	4.15494
	2.00	15	12.4000	9.52290	2.45880
Round48h	1.00	15	25.4667	19.23489	4.96643
	2.00	15	35.2000	18.52489	4.78311

24h interval

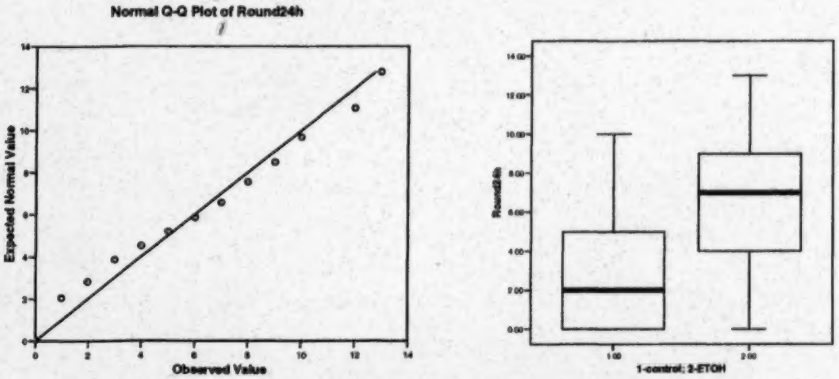


Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

Page

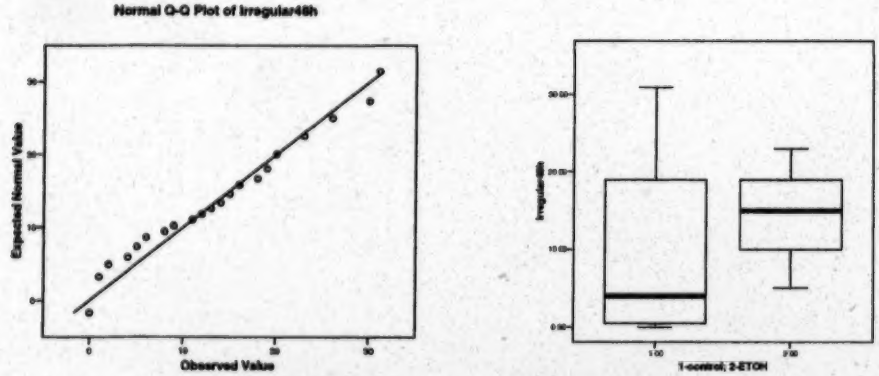


Markedly skewed data in the control group (Kolmogorov-Smirnov Test for Normality, $p<0.05$),, unequal variance (Levene's Test for Equality of Variance $p<0.05$)

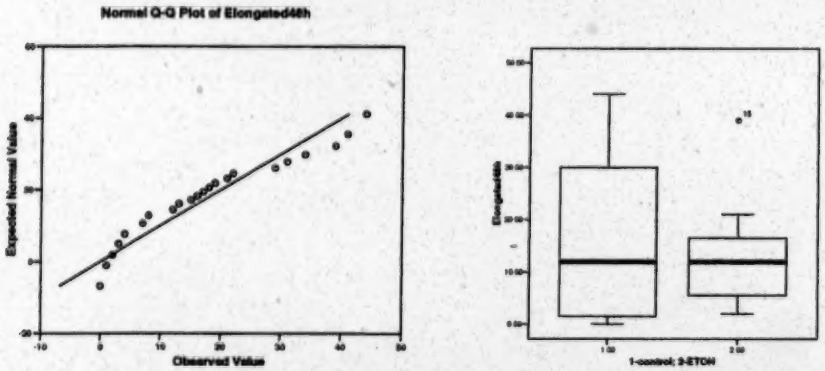


Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

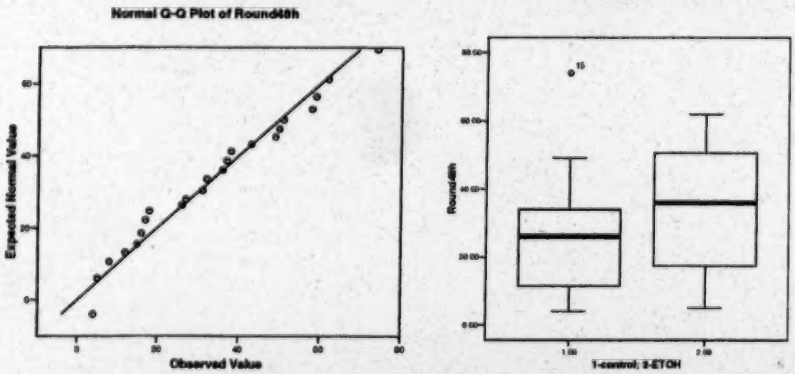
Page
48h interval



Markedly skewed data in the control group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), equal variance (Levene's Test for Equality of Variance $p<0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Irregular24h	1.00	15	15.53	233.00
	2.00	15	15.47	232.00
	Total	30		
Elongated24h	1.00	15	14.57	218.50
	2.00	15	16.43	246.50
	Total	30		
Round24h	1.00	15	11.97	179.50
	2.00	15	19.03	285.50
	Total	30		
Irregular48h	1.00	15	13.00	195.00
	2.00	15	18.00	270.00
	Total	30		
Elongated48h	1.00	15	15.57	233.50
	2.00	15	15.43	231.50
	Total	30		
Round48h	1.00	15	12.80	192.00
	2.00	15	18.20	273.00
	Total	30		

Test Statistics[b]

	Irregular24h	Elongated24h	Round24h	Irregular48h	Elongated48h	Round48h
Mann-Whitney U	112.000	98.500	59.500	75.000	111.500	72.000
Wilcoxon W	232.000	218.500	179.500	195.000	231.500	192.000
Z	-.021	-.581	-2.217	-1.558	-.042	-1.681
Asymp. Sig. (2-tailed)	.983	.561	.027	.119	.967	.093
Exact Sig. [2*(1-tailed Sig.)]	1.000[a]	.567[a]	.026[a]	.126[a]	.967[a]	.098[a]

a. Not corrected for ties.

b. Grouping Variable: 1-control; 2-ETOH

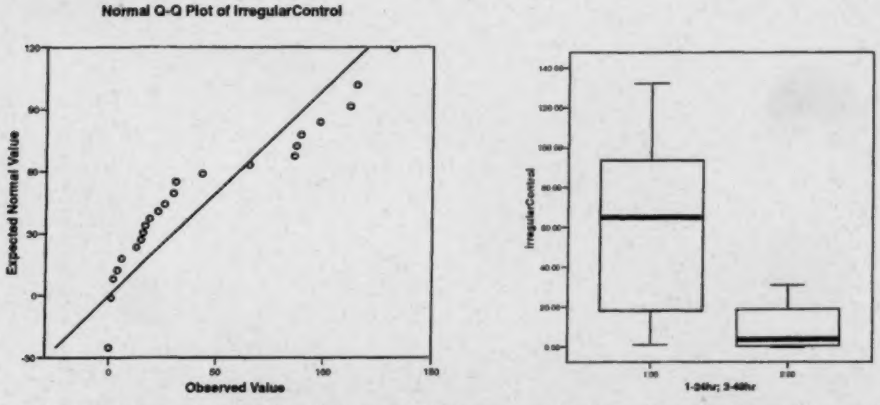
Page

2.2.2 *Comparison of control and alcohol-treated trunk neural crest cells between intervals*

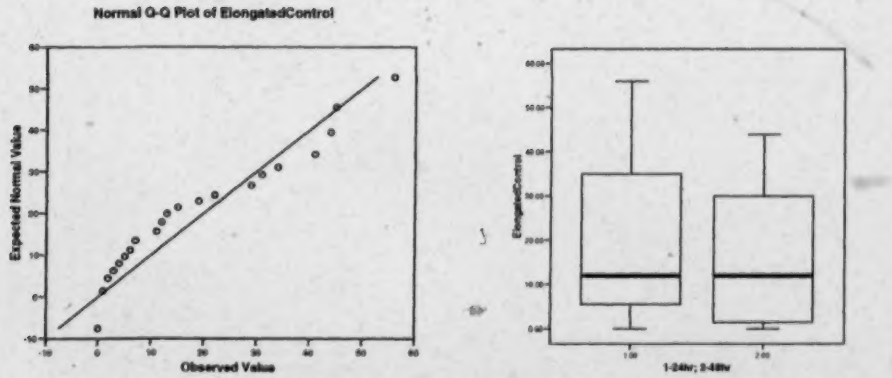
Group Statistics

	1-24hr; 2-48hr	N	Mean	Std. Deviation	Std. Error Mean
IrregularControl	1.00	15	61.0667	44.45136	11.47729
	2.00	15	10.1333	11.88557	3.06884
ElongatedControl	1.00	15	19.2667	18.58750	4.79927
	2.00	15	16.3333	16.09200	4.15494
RoundControl	1.00	15	3.0667	3.15021	.81338
	2.00	15	25.4667	19.23489	4.96643
IrregularETOH	1.00	15	59.4667	19.65730	5.07549
	2.00	15	14.2667	5.72547	1.47831
ElongatedETOH	1.00	15	18.8000	11.14963	2.87882
	2.00	15	12.4000	9.52290	2.45880
RoundETOH	1.00	15	6.3333	4.09994	1.05860
	2.00	15	35.2000	18.52489	4.78311

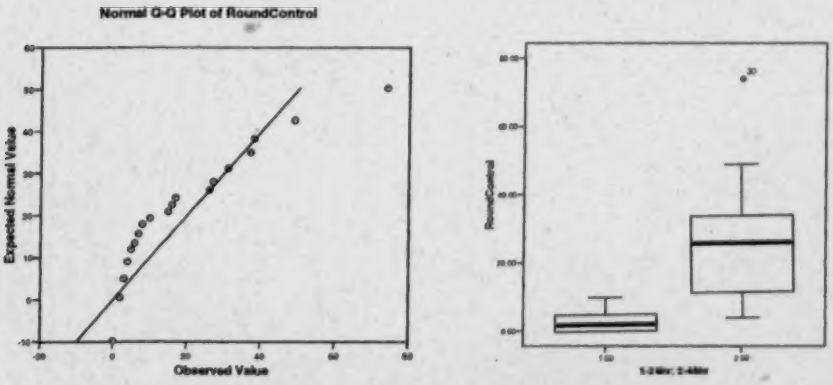
Control group



Markedly skewed data in the 48h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)



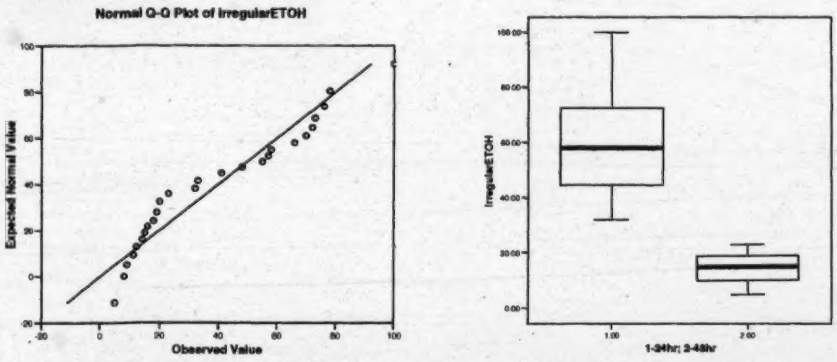
Markedly skewed data in the 24h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$),, equal variance (Levene's Test for Equality of Variance $p>0.05$)



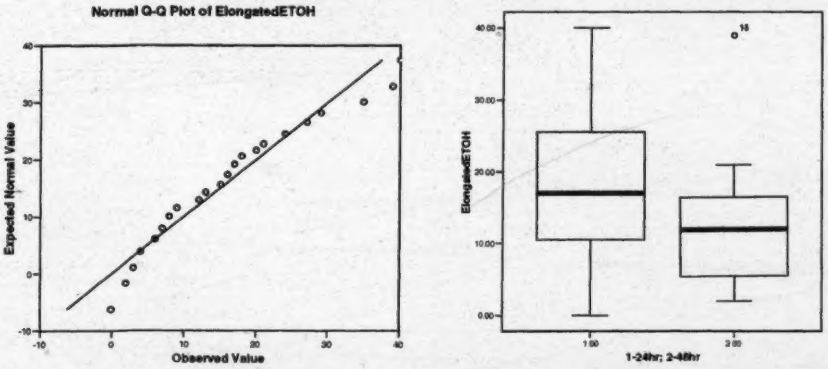
Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

Page

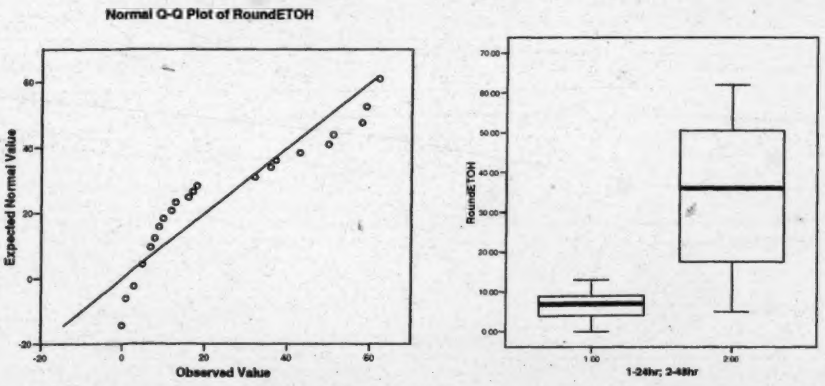
ETOH group



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-24hr; 2-48hr	N	Mean Rank	Sum of Ranks
IrregularControl	1.00	15	21.00	315.00
	2.00	15	10.00	150.00
	Total	30		
ElongatedControl	1.00	15	16.57	248.50
	2.00	15	14.43	216.50
	Total	30		
RoundControl	1.00	15	8.93	134.00
	2.00	15	22.07	331.00
	Total	30		
IrregularETOH	1.00	15	23.00	345.00
	2.00	15	8.00	120.00
	Total	30		
ElongatedETOH	1.00	15	18.30	274.50
	2.00	15	12.70	190.50
	Total	30		
RoundETOH	1.00	15	8.73	131.00
	2.00	15	22.27	334.00
	Total	30		

Test Statistics[b]

	Irregular Control	Elongated Control	RoundControl	IrregularETOH	Elongated ETOH	RoundETOH
Mann-Whitney U	30.000	96.500	14.000	.000	70.500	11.000
Wilcoxon W	150.000	216.500	134.000	120.000	190.500	131.000
Z	-3.428	-.665	-4.100	-4.668	-1.744	-4.217
Asymp. Sig. (2-tailed)	.001	.506	.000	.000	.081	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[a]	.512[a]	.000[a]	.000[a]	.081[a]	.000[a]

a. Not corrected for ties.

b. Grouping Variable: 1-24hr; 2-48hr

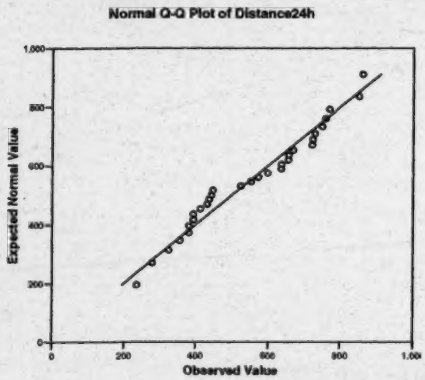
Page

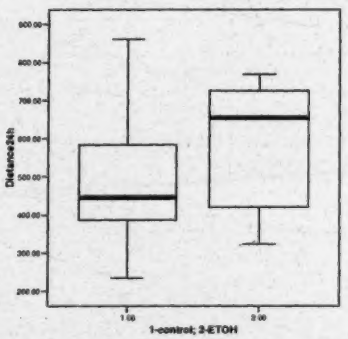
2.3 Trunk neural crest cells / Fibronectin / Danilchik's medium / Distance

2.3.1 Comparison between control and alcohol-treated trunk neural crest cells

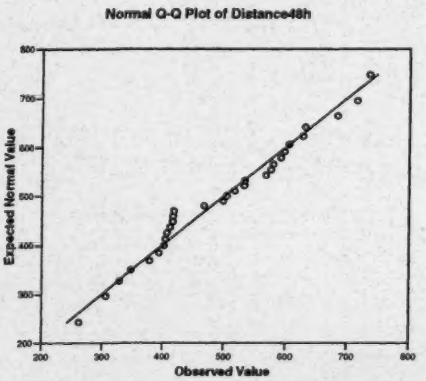
Group Statistics

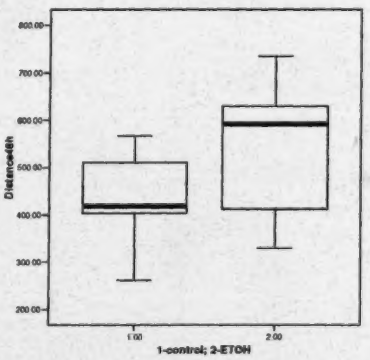
	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Distance24h	1.00	15	505.5627	183.23507	47.31109
	2.00	15	598.7786	160.95288	41.55786
Distance48h	1.00	15	439.1812	88.68499	22.89836
	2.00	15	551.0350	131.26153	33.89158





Markedly skewed data in the ETOH group (Kolmogorov-Smirnov Test for Normality, $p<0.05$),, equal variance (Levene's Test for Equality of Variance $p>0.05$)





Markedly skewed data in the ETOH group (Kolmogorov-Smirnov Test for Normality, $p<0.05$),, equal variance (Levene's Test for Equality of Variance $p>0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Distance24h	1.00	15	13.27	199.00
	2.00	15	17.73	266.00
	Total	30		
Distance48h	1.00	15	11.73	176.00
	2.00	15	19.27	289.00
	Total	30		

Test Statistics[b]

	Distance24h	Distance48h
Mann-Whitney U	79.000	56.000
Wilcoxon W	199.000	176.000
Z	-1.390	-2.344
Asymp. Sig. (2-tailed)	.165	.019
Exact Sig. [2*(1-tailed Sig.)]	.174[a]	.019[a]

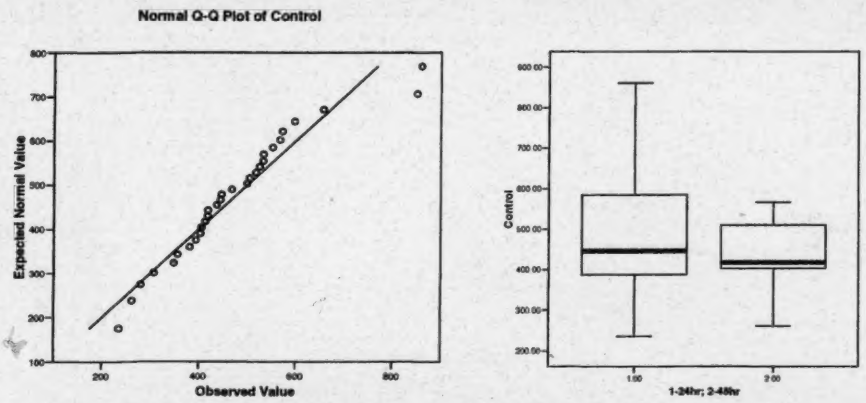
a. Not corrected for ties.

b. Grouping Variable: 1-control; 2-ETOH

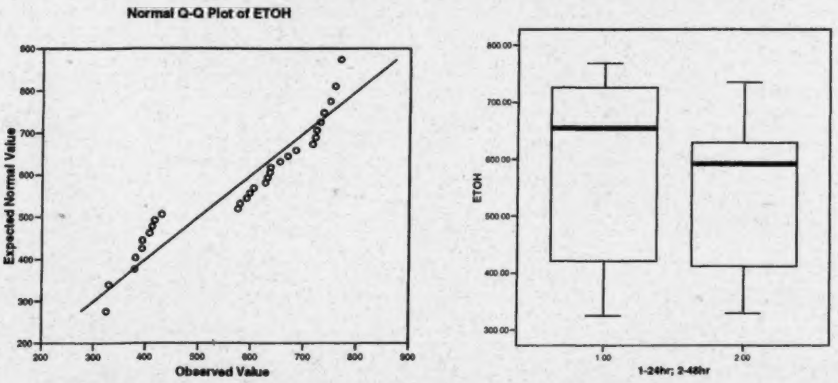
2.3.2 *Comparison of control and alcohol-treated trunk neural crest cells between intervals*

Group Statistics

	1-24hr; 2-48hr	N	Mean	Std. Deviation	Std. Error Mean
Control	1.00	15	505.5627	183.23507	47.31109
	2.00	15	439.1812	88.68499	22.89836
ETOH	1.00	15	598.7786	160.95288	41.55786
	2.00	15	551.0350	131.26153	33.89158



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)



Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-24hr; 2-48hr	N	Mean Rank	Sum of Ranks
Control	1.00	15	17.00	255.00
	2.00	15	14.00	210.00
	Total	30		
ETOH	1.00	15	17.87	268.00
	2.00	15	13.13	197.00
	Total	30		

Test Statistics[b]

	Control	ETOH
Mann-Whitney U	90.000	77.000
Wilcoxon W	210.000	197.000
Z	-.933	-1.472
Asymp. Sig. (2-tailed)	.351	.141
Exact Sig. [2*(1-tailed Sig.)]	.367[a]	.148[a]

a. Not corrected for ties.

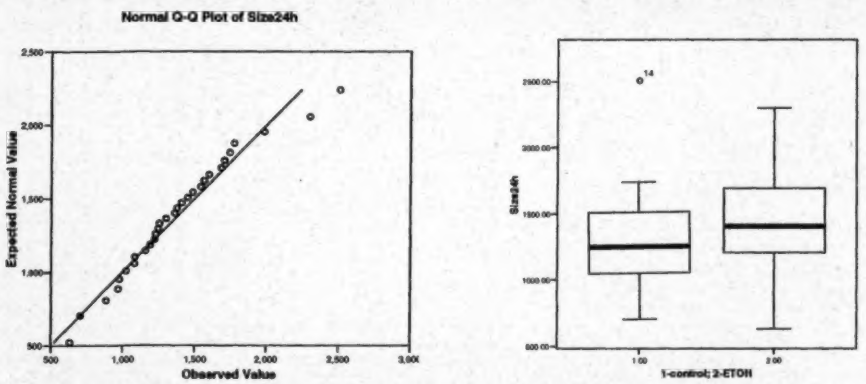
b. Grouping Variable: 1-24hr; 2-48hr

2.4 Trunk neural crest cells / Fibronectin / Danilchik's medium / Size

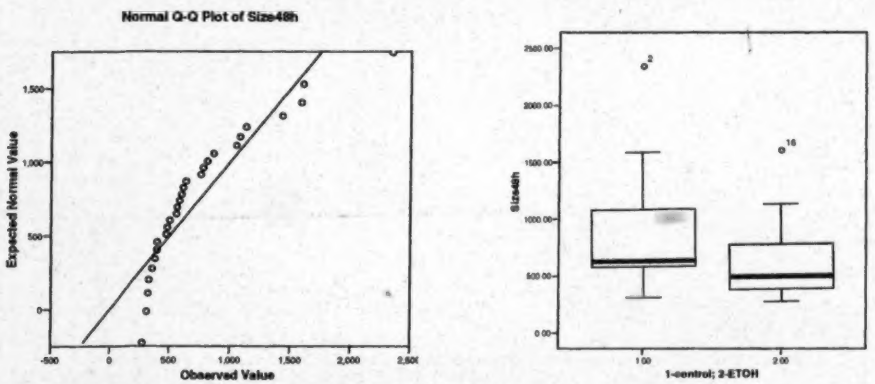
2.4.1 Comparison between control and alcohol-treated trunk neural crest cells

Group Statistics

	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Size24h	1.00	15	1316.9009	432.53609	111.68034
	2.00	15	1446.7299	413.18685	106.68439
Size48h	1.00	13	890.3994	587.80040	163.02650
	2.00	15	645.6247	369.26664	95.34424



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Size24h	1.00	15	13.73	206.00
	2.00	15	17.27	259.00
	Total	30		
Size48h	1.00	13	16.77	218.00
	2.00	15	12.53	188.00
	Total	28		

Test Statistics[b]

	Size24h	Size48h
Mann-Whitney U	86.000	68.000
Wilcoxon W	206.000	188.000
Z	-1.099	-1.359
Asymp. Sig. (2-tailed)	.272	.174
Exact Sig. [2*(1-tailed Sig.)]	.285[a]	.185[a]

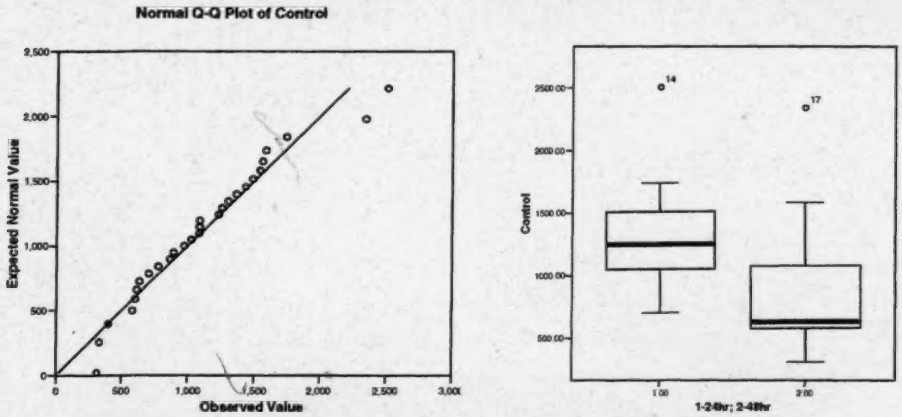
a. Not corrected for ties.

b. Grouping Variable: 1-control; 2-ETOH

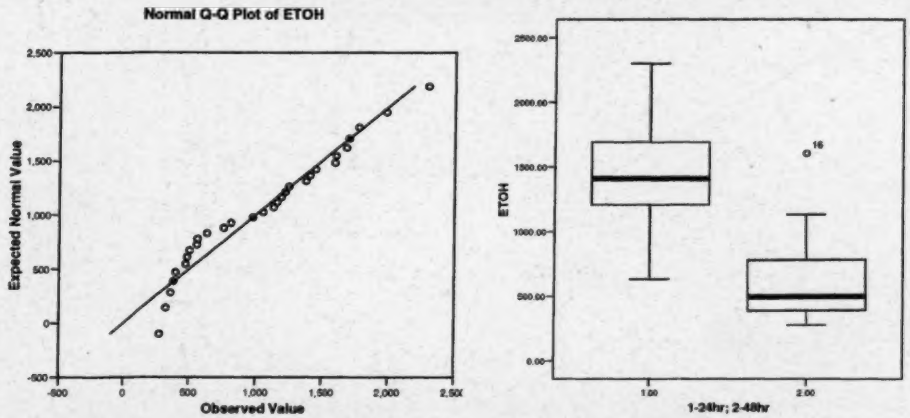
2.4.2 *Comparison of control and alcohol-treated trunk neural crest cells between intervals*

Group Statistics

	1-24hr; 2-48hr	N	Mean	Std. Deviation	Std. Error Mean
Control	1.00	15	1316.9009	432.53609	111.68034
	2.00	13	890.3994	587.80040	163.02650
ETOH	1.00	15	1446.7299	413.18685	106.68439
	2.00	15	645.6247	369.26664	95.34424



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-24hr; 2-48hr	N	Mean Rank	Sum of Ranks
Control	1.00	15	18.07	271.00
	2.00	13	10.38	135.00
	Total	28		
ETOH	1.00	15	21.93	329.00
	2.00	15	9.07	136.00
	Total	30		

Test Statistics[b]

	Control	ETOH
Mann-Whitney U	44.000	16.000
Wilcoxon W	135.000	136.000
Z	-2.464	-4.003
Asymp. Sig. (2-tailed)	.014	.000
Exact Sig. [2*(1-tailed Sig.)]	.013[a]	.000[a]

a. Not corrected for ties.

b. Grouping Variable: 1-24hr; 2-48hr

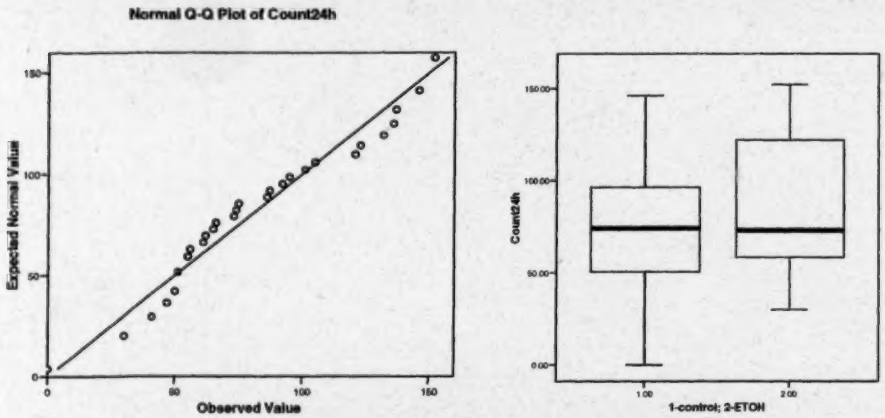
Page

3.1 Trunk neural crest cells / Collagen / Danilchik's medium / Count

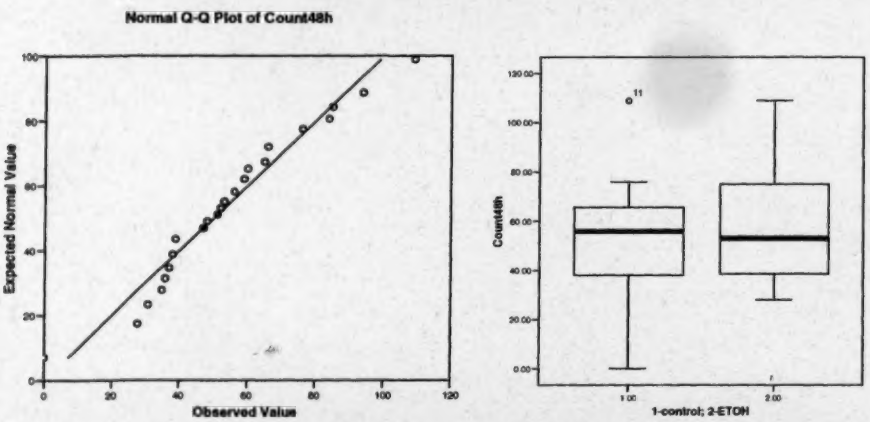
3.1.1 Comparison between control and alcohol-treated trunk neural crest cells

Group Statistics

	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Count24h	1.00	15	75.8667	38.03357	9.82023
	2.00	15	85.5333	37.85473	9.77405
Count48h	1.00	15	53.2000	24.38149	6.29527
	2.00	15	58.9333	24.05490	6.21095



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Count24h	1.00	15	14.53	218.00
	2.00	15	16.47	247.00
	Total	30		
Count48h	1.00	15	14.97	224.50
	2.00	15	16.03	240.50
	Total	30		

Test Statistics[b]

	Count24h	Count48h
Mann-Whitney U	98.000	104.500
Wilcoxon W	218.000	224.500
Z	-.602	-.332
Asymp. Sig. (2-tailed)	.547	.740
Exact Sig. [2*(1-tailed Sig.)]	.567[a]	.744[a]

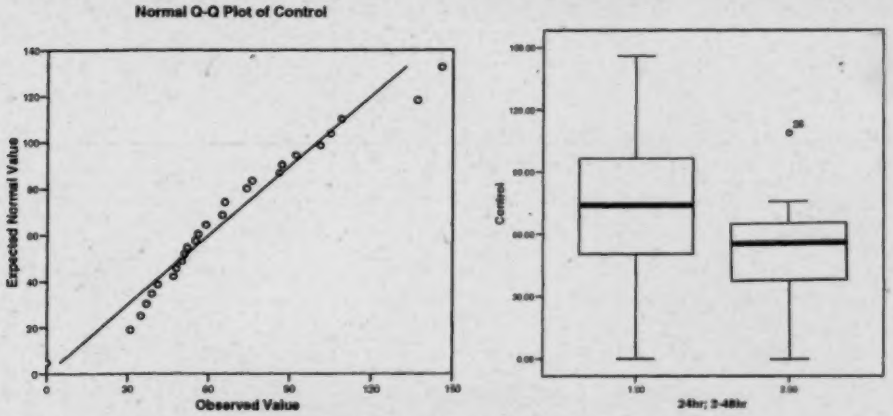
a. Not corrected for ties.

b. Grouping Variable: 1-control; 2-ETOH

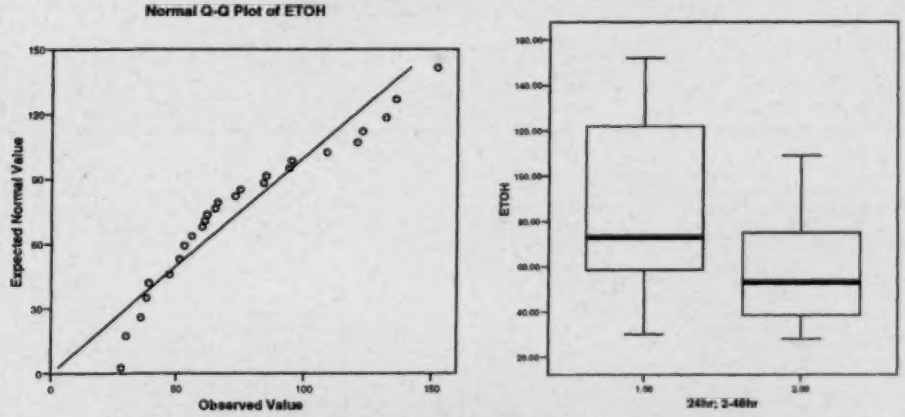
3.1.2 Comparison of control and alcohol-treated trunk neural crest cells between intervals

Group Statistics

	24hr; 2-48hr	N	Mean	Std. Deviation	Std. Error Mean
Control	1.00	15	75.8667	38.03357	9.82023
	2.00	15	53.2000	24.38149	6.29527
ETOH	1.00	15	85.5333	37.85473	9.77405
	2.00	15	58.9333	24.05490	6.21095



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), unequal variance (Levene's Test for Equality of Variance p<0.05)

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	24hr; 2-48hr	N	Mean Rank	Sum of Ranks
Control	1.00	15	18.30	274.50
	2.00	15	12.70	190.50
	Total	30		
ETOH	1.00	15	18.90	283.50
	2.00	15	12.10	181.50
	Total	30		

Test Statistics[b]

	Control	ETOH
Mann-Whitney U	70.500	61.500
Wilcoxon W	190.500	181.500
Z	-1.743	-2.117
Asymp. Sig. (2-tailed)	.081	.034
Exact Sig. [2*(1-tailed Sig.)]	.081[a]	.033[a]

a. Not corrected for ties.

b. Grouping Variable: 24hr; 2-48hr

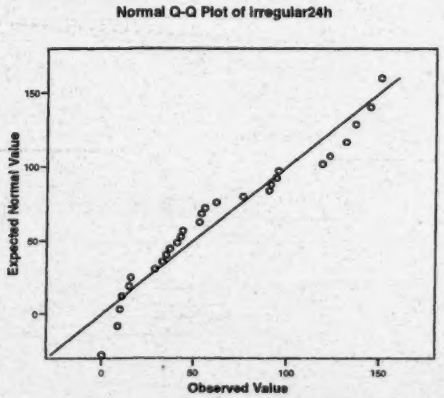
3.2 Trunk neural crest cells / Collagen / Danilchik's medium / Shape

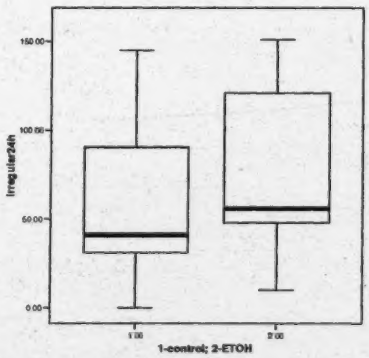
3.2.1 Comparison between control and alcohol-treated trunk neural crest cells

Group Statistics

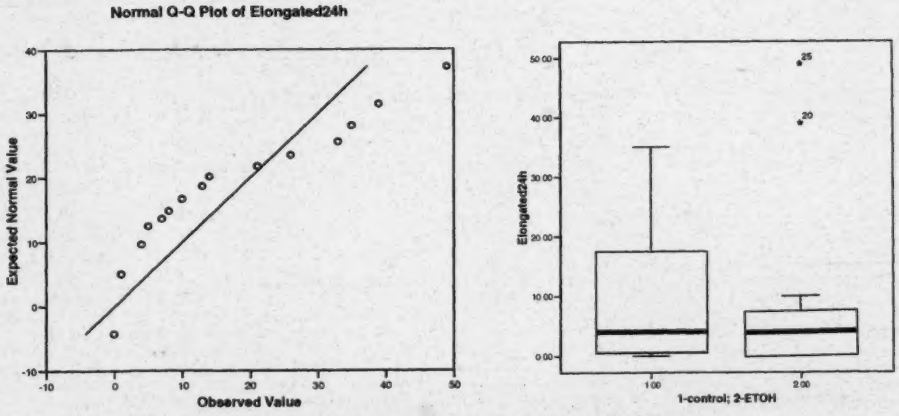
	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Irregular24h	1.00	15	58.2000	45.12712	11.65177
	2.00	15	74.2000	47.15809	12.17617
Elongated24h	1.00	15	10.8000	12.47970	3.22224
	2.00	15	8.5333	14.88464	3.84320
Round24h	1.00	15	6.8667	9.78239	2.52580
	2.00	15	2.8000	3.80225	.98174
Irregular48h	1.00	15	31.8667	26.11477	6.74280
	2.00	15	34.3333	22.76171	5.87705
Elongated48h	1.00	15	8.6667	10.38314	2.68091
	2.00	15	9.8000	8.95385	2.31187
Round48h	1.00	15	12.6667	9.70763	2.50650
	2.00	15	14.8000	8.88980	2.29534

24h interval

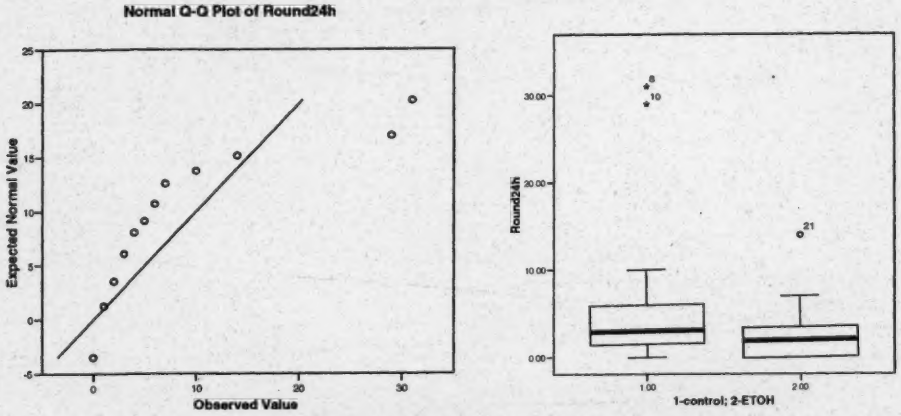




Markedly skewed data in the control group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

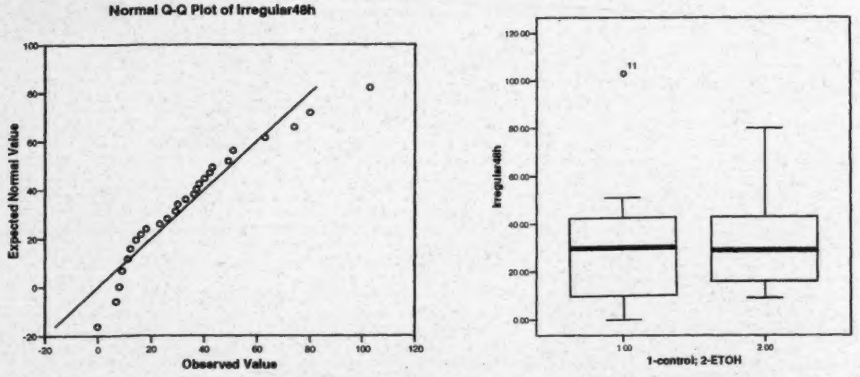


Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

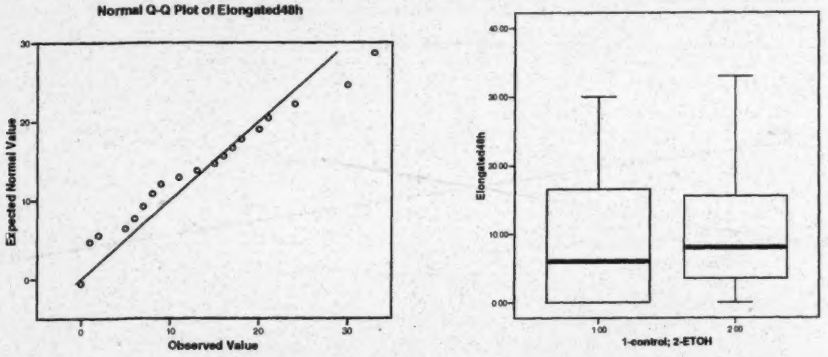


Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

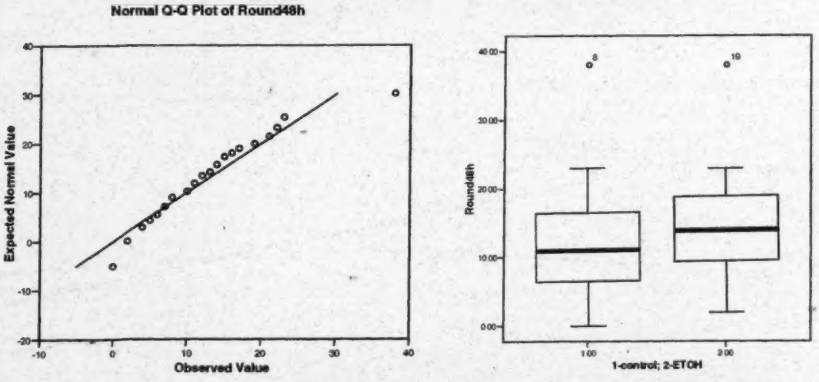
Page
48h interval



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)



Markedly skewed data in the control group (Kolmogorov-Smirnov Test for Normality, p<0.05), equal variance (Levene's Test for Equality of Variance p>0.05)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Irregular24h	1.00	15	13.67	205.00
	2.00	15	17.33	260.00
	Total	30		
Elongated24h	1.00	15	16.57	248.50
	2.00	15	14.43	216.50
	Total	30		
Round24h	1.00	15	17.73	266.00
	2.00	15	13.27	199.00
	Total	30		
Irregular48h	1.00	15	14.93	224.00
	2.00	15	16.07	241.00
	Total	30		
Elongated48h	1.00	15	14.50	217.50
	2.00	15	16.50	247.50
	Total	30		
Round48h	1.00	15	14.07	211.00
	2.00	15	16.93	254.00
	Total	30		

Test Statistics[b]

	Irregular24h	Elongated24h	Round24h	Irregular48h	Elongated48h	Round48h
Mann-Whitney U	85.000	96.500	79.000	104.000	97.500	91.000
Wilcoxon W	205.000	216.500	199.000	224.000	217.500	211.000
Z	-1.141	-.674	-1.407	-.353	-.631	-.893
Asymp. Sig. (2-tailed)	.254	.500	.159	.724	.528	.372
Exact Sig. [2*(1-tailed Sig.)]	.267[a]	.512[a]	.174[a]	.744[a]	.539[a]	.389[a]

a. Not corrected for ties.

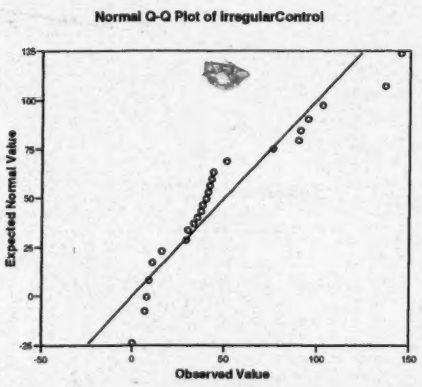
b. Grouping Variable: 1-control; 2-ETOH

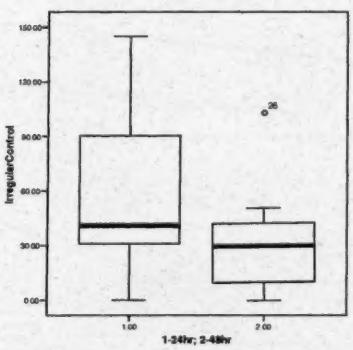
3.2.2 Comparison of control and alcohol-treated trunk neural crest cells between intervals

Group Statistics

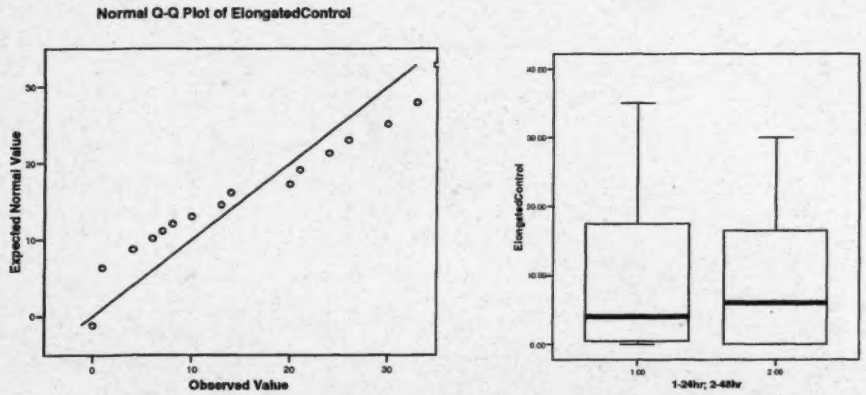
	1-24hr; 2-48hr	N	Mean	Std. Deviation	Std. Error Mean
IrregularControl	1.00	15	58.2000	45.12712	11.65177
	2.00	15	31.8667	26.11477	6.74280
ElongatedControl	1.00	15	10.8000	12.47970	3.22224
	2.00	15	8.6667	10.38314	2.68091
RoundControl	1.00	15	6.8667	9.78239	2.52580
	2.00	15	12.6667	9.70763	2.50650
IrregularETOH	1.00	15	74.2000	47.15809	12.17617
	2.00	15	34.3333	22.76171	5.87705
ElongatedETOH	1.00	15	8.5333	14.88464	3.84320
	2.00	15	9.8000	8.95385	2.31187
RoundETOH	1.00	15	2.8000	3.80225	.98174
	2.00	15	14.8000	8.88980	2.29534

Control group

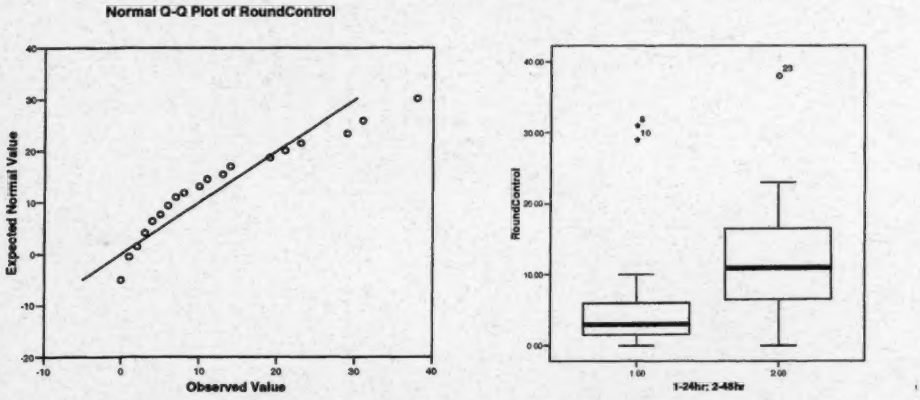




Markedly skewed data in the 24h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$),, unequal variance (Levene's Test for Equality of Variance $p<0.05$)

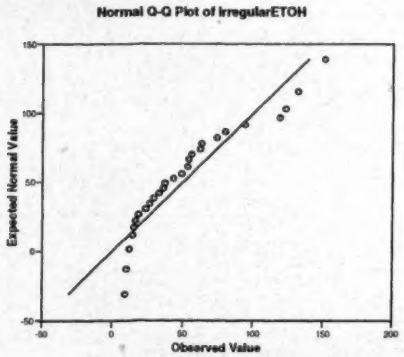


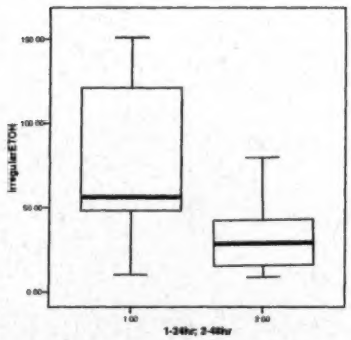
Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)



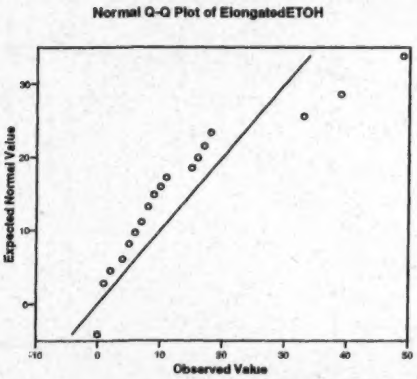
Markedly skewed data in the 24h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

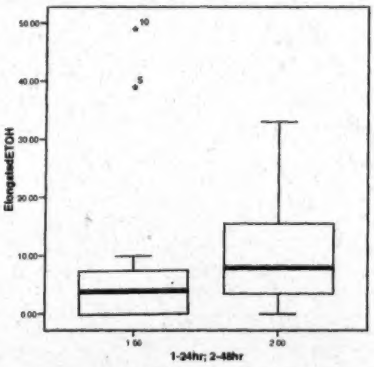
Page
ETOH group



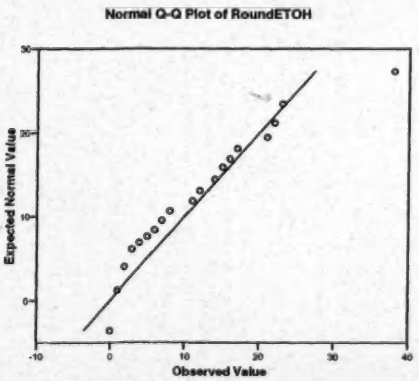


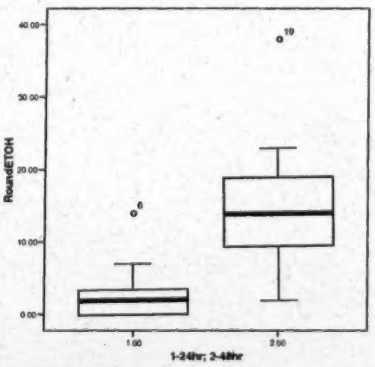
Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)





Markedly skewed data in the 24h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)





Markedly skewed data in the 24h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-24hr; 2-48hr	N	Mean Rank	Sum of Ranks
IrregularControl	1.00	15	17.80	267.00
	2.00	15	13.20	198.00
	Total	30		
ElongatedControl	1.00	15	16.47	247.00
	2.00	15	14.53	218.00
	Total	30		
RoundControl	1.00	15	11.80	177.00
	2.00	15	19.20	288.00
	Total	30		
IrregularETOH	1.00	15	19.33	290.00
	2.00	15	11.67	175.00
	Total	30		
ElongatedETOH	1.00	15	13.27	199.00
	2.00	15	17.73	266.00
	Total	30		
RoundETOH	1.00	15	9.03	135.50
	2.00	15	21.97	329.50
	Total	30		

Test Statistics[b]

	Irregular Control	Elongated Control	RoundControl	IrregularETOH	Elongated ETOH	RoundETOH
Mann-Whitney U	78.000	98.000	57.000	55.000	79.000	15.500
Wilcoxon W	198.000	218.000	177.000	175.000	199.000	135.500
Z	-1.432	-.613	-2.308	-2.386	-1.403	-4.039
Asymp. Sig. (2-tailed)	.152	.540	.021	.017	.160	.000
Exact Sig. [2*(1-tailed Sig.)]	.161[a]	.567[a]	.021[a]	.016[a]	.174[a]	.000[a]

a. Not corrected for ties.

b. Grouping Variable: 1-24hr; 2-48hr

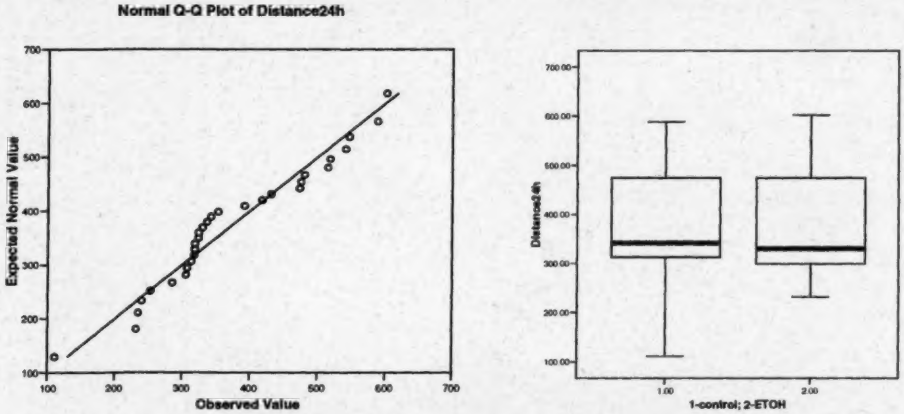
Page

3.3 Trunk neural crest cells / Collagen / Danilchik's medium / Distance

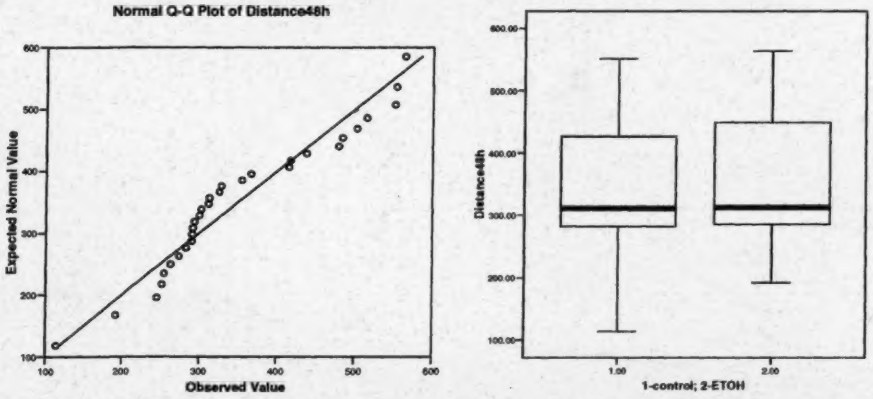
3.3.1 Comparison between control and alcohol-treated trunk neural crest cells

Group Statistics

	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Distance24h	1.00	15	371.2785	121.43651	31.35477
	2.00	15	377.0234	121.93986	31.48474
Distance48h	1.00	15	346.1627	116.58233	30.10143
	2.00	15	358.0163	116.01505	29.95496



Markedly skewed data in the ETOH group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)



Markedly skewed data in the ETOH group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Distance24h	1.00	15	15.73	236.00
	2.00	15	15.27	229.00
	Total	30		
Distance48h	1.00	15	15.07	226.00
	2.00	15	15.93	239.00
	Total	30		

Test Statistics[b]

	Distance24h	Distance48h
Mann-Whitney U	109.000	106.000
Wilcoxon W	229.000	226.000
Z	-.145	-.270
Asymp. Sig. (2-tailed)	.885	.787
Exact Sig. [2*(1-tailed Sig.)]	.902[a]	.806[a]

a. Not corrected for ties.

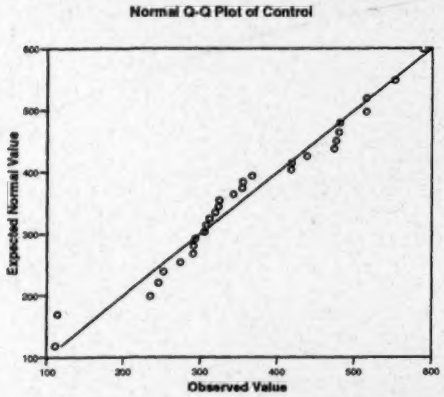
b. Grouping Variable: 1-control; 2-ETOH

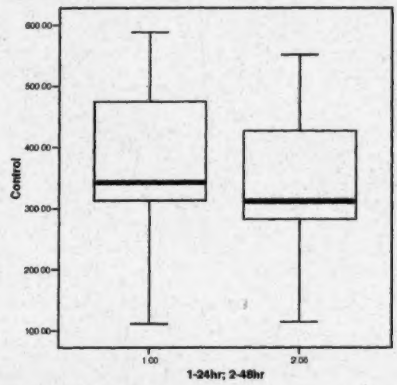
Page

3.3.2 *Comparison of control and alcohol-treated trunk neural crest cells between intervals*

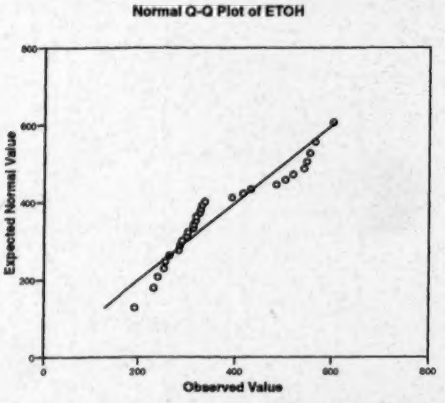
Group Statistics

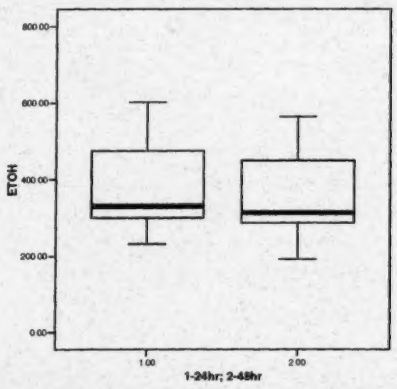
	1-24hr; 2-48hr	N	Mean	Std. Deviation	Std. Error Mean
Control	1.00	15	371.2785	121.43651	31.35477
	2.00	15	346.1627	116.58233	30.10143
ETOH	1.00	15	377.0234	121.93986	31.48474
	2.00	15	358.0163	116.01505	29.95496





Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)





Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-24hr; 2-48hr	N	Mean Rank	Sum of Ranks
Control	1.00	15	16.73	251.00
	2.00	15	14.27	214.00
	Total	30		
ETOH	1.00	15	16.40	246.00
	2.00	15	14.60	219.00
	Total	30		

Test Statistics[b]

	Control	ETOH
Mann-Whitney U	94.000	99.000
Wilcoxon W	214.000	219.000
Z	-.767	-.560
Asymp. Sig. (2-tailed)	.443	.576
Exact Sig. [2*(1-tailed Sig.)]	.461[a]	.595[a]

a. Not corrected for ties.

b. Grouping Variable: 1-24hr; 2-48hr

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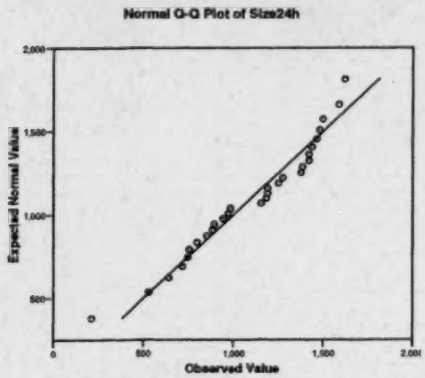
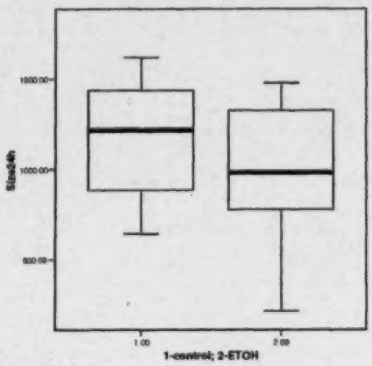
3.4 Trunk neural crest cells / Collagen / Danilchik's medium / Size

3.4.1 Comparison between control and alcohol-treated trunk neural crest cells

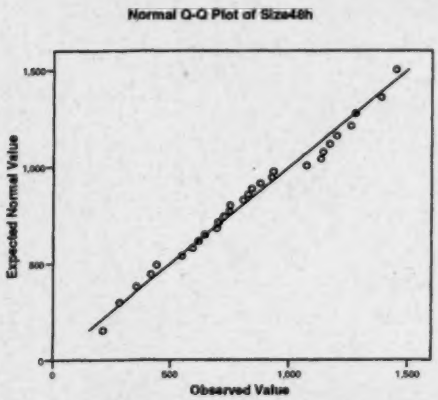
Group Statistics

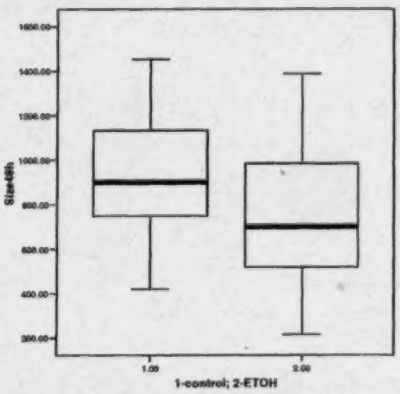
	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Size24h	1.00	14	1188.9352	327.63201	87.56334
	2.00	15	1014.0584	367.55100	94.90126
Size48h	1.00	14	923.7085	292.37617	78.14082
	2.00	15	740.3576	353.53057	91.28120





Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)





Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Size24h	1.00	14	17.00	238.00
	2.00	15	13.13	197.00
	Total	29		
Size48h	1.00	14	17.57	246.00
	2.00	15	12.60	189.00
	Total	29		

Test Statistics[b]

	Size24h	Size48h
Mann-Whitney U	77.000	69.000
Wilcoxon W	197.000	189.000
Z	-1.222	-1.571
Asymp. Sig. (2-tailed)	.222	.116
Exact Sig. [2*(1-tailed Sig.)]	.234[a]	.123[a]

a. Not corrected for ties.

b. Grouping Variable: 1-control; 2-ETOH

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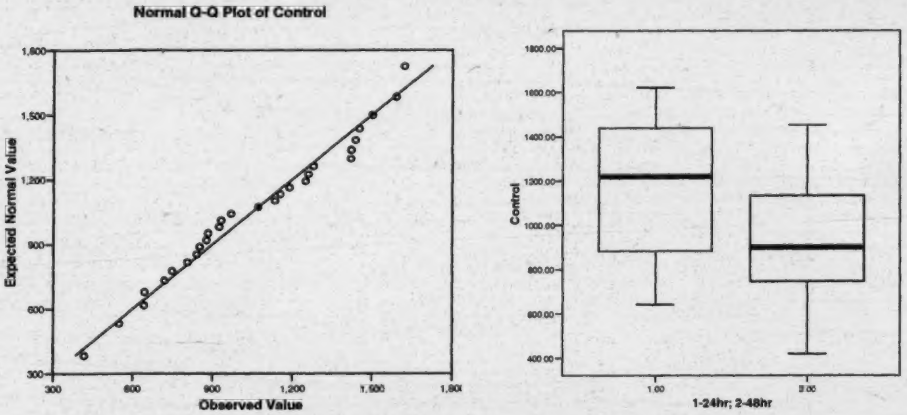
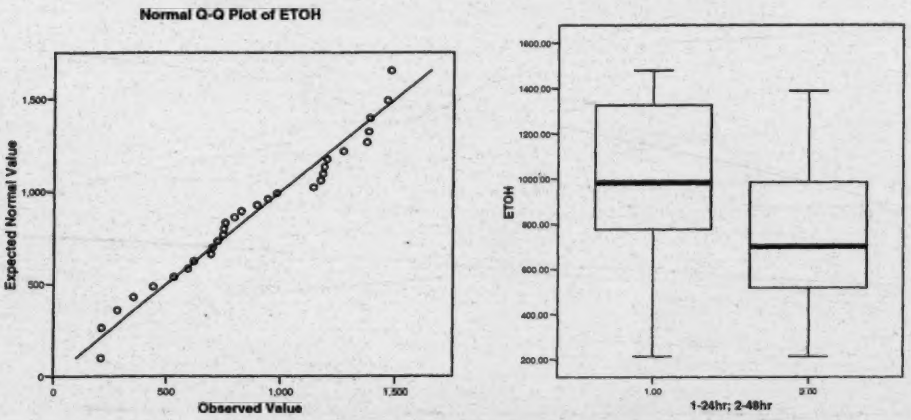
3.4.2 Comparison of control and alcohol-treated trunk neural crest cells between intervals

Group Statistics

	1-24hr; 2-48hr	N	Mean	Std. Deviation	Std. Error Mean
Control	1.00	14	1188.9352	327.63201	87.56334
	2.00	14	923.7085	292.37617	78.14082
ETOH	1.00	15	1014.0584	367.55100	94.90126
	2.00	15	740.3576	353.53057	91.28120



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-24hr; 2-48hr	N	Mean Rank	Sum of Ranks
Control	1.00	14	17.57	246.00
	2.00	14	11.43	160.00
	Total	28		
ETOH	1.00	15	18.93	284.00
	2.00	15	12.07	181.00
	Total	30		

Test Statistics[b]

	Control	ETOH
Mann-Whitney U	55.000	61.000
Wilcoxon W	160.000	181.000
Z	-1.976	-2.136
Asymp. Sig. (2-tailed)	.048	.033
Exact Sig. [2*(1-tailed Sig.)]	.050[a]	.033[a]

a. Not corrected for ties.

b. Grouping Variable: 1-24hr; 2-48hr

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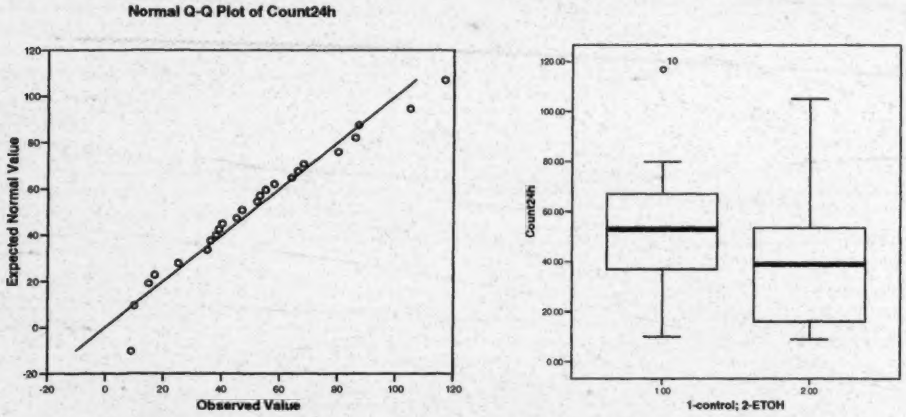
4.1 Trunk neural crest cells / Collagen / Enriched medium / Count

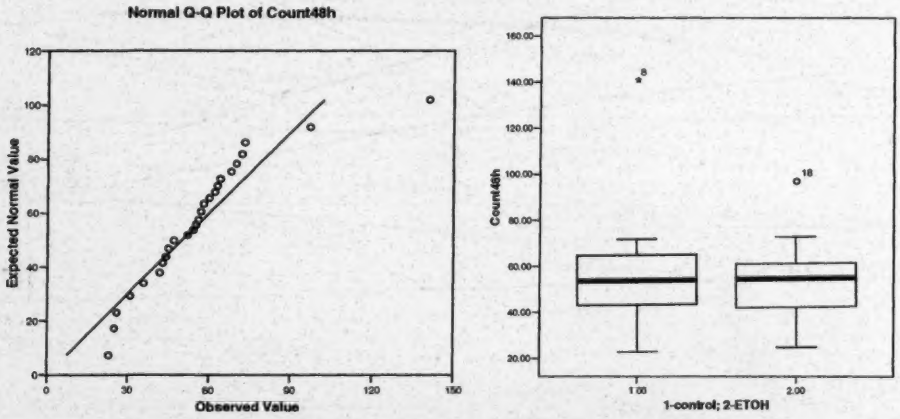
4.1.1 Comparison between control and alcohol-treated trunk neural crest cells

Group Statistics

	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Count24h	1.00	15	55.4667	25.51433	6.58777
	2.00	15	41.4667	30.82408	7.95874
Count48h	1.00	15	56.2667	27.93990	7.21405
	2.00	15	53.0000	18.01983	4.65270



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)



Markedly skewed distribution in the control group (Kolmogorov-Smirnov Test for Normality, p<0.05),, equal variance (Levene's Test for Equality of Variance p>0.05)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Count24h	1.00	15	17.77	266.50
	2.00	15	13.23	198.50
	Total	30		
Count48h	1.00	15	15.57	233.50
	2.00	15	15.43	231.50
	Total	30		

Test Statistics[b]

	Count24h	Count48h
Mann-Whitney U	78.500	111.500
Wilcoxon W	198.500	231.500
Z	-1.412	-.041
Asymp. Sig. (2-tailed)	.158	.967
Exact Sig. [2*(1-tailed Sig.)]	.161[a]	.967[a]

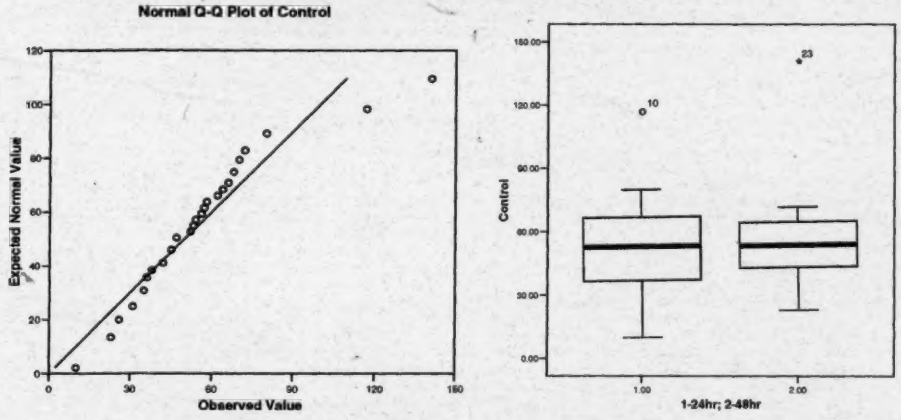
a. Not corrected for ties.

b. Grouping Variable: 1-control; 2-ETOH

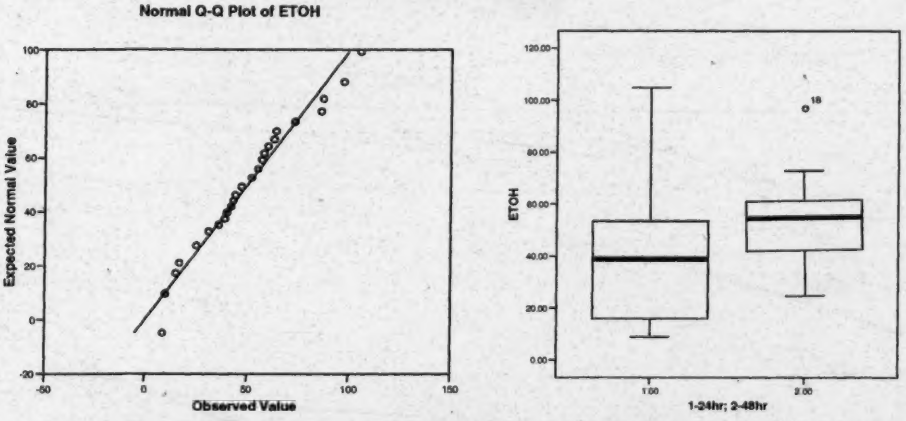
4.1.2 Comparison of control and alcohol-treated trunk neural crest cells between intervals

Group Statistics

	1-24hr; 2-48hr	N	Mean	Std. Deviation	Std. Error Mean
Control	1.00	15	55.4667	25.51433	6.58777
	2.00	15	56.2667	27.93990	7.21405
ETOH	1.00	15	41.4667	30.82408	7.95874
	2.00	15	53.0000	18.01983	4.65270



Markedly skewed data in the 48h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p<0.05$)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

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NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-24hr; 2-48hr	N	Mean Rank	Sum of Ranks
Control	1.00	15	15.70	235.50
	2.00	15	15.30	229.50
	Total	30		
ETOH	1.00	15	12.73	191.00
	2.00	15	18.27	274.00
	Total	30		

Test Statistics[b]

	Control	ETOH
Mann-Whitney U	109.500	71.000
Wilcoxon W	229.500	191.000
Z	-.125	-1.723
Asymp. Sig. (2-tailed)	.901	.085
Exact Sig. [2*(1-tailed Sig.)]	.902[a]	.089[a]

a. Not corrected for ties.

b. Grouping Variable: 1-24hr; 2-48hr

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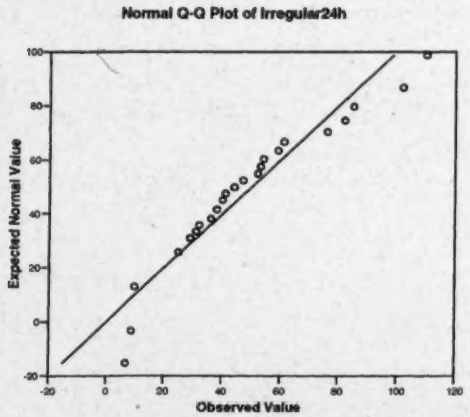
4.2 Trunk neural crest cells / Collagen / Enriched medium / Shape

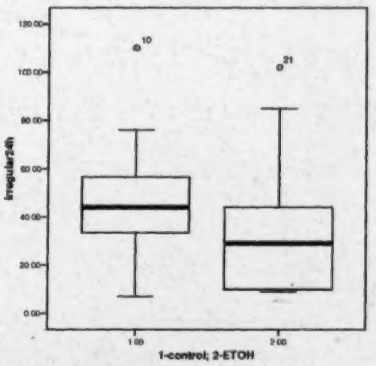
4.2.1 Comparison between control and alcohol-treated trunk neural crest cells

Group Statistics

	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Irregular24h	1.00	15	46.4000	25.66766	6.62736
	2.00	15	37.0000	30.17568	7.79133
Elongated24h	1.00	15	2.4000	1.91982	.49570
	2.00	15	1.8000	2.45531	.63396
Round24h	1.00	15	7.4000	8.87855	2.29243
	2.00	15	2.6667	2.94392	.76012
Irregular48h	1.00	15	47.9333	26.72203	6.89960
	2.00	15	43.9333	17.58842	4.54131
Elongated48h	1.00	15	.9333	1.86956	.48272
	2.00	15	.6667	1.04654	.27021
Round48h	1.00	15	7.4000	6.40089	1.65270
	2.00	15	8.6667	7.00680	1.80915

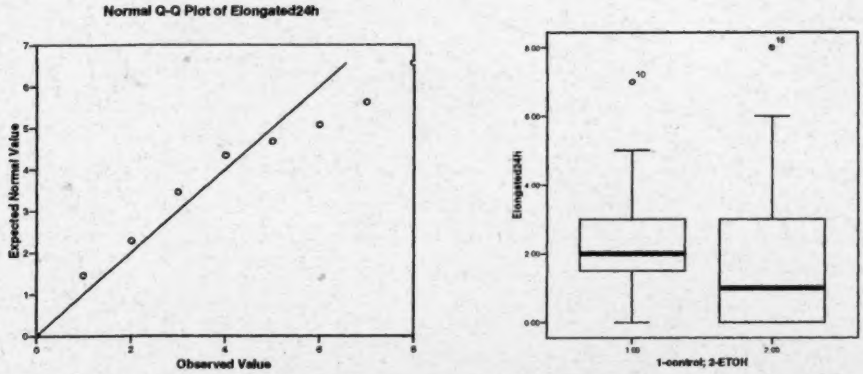
24h interval



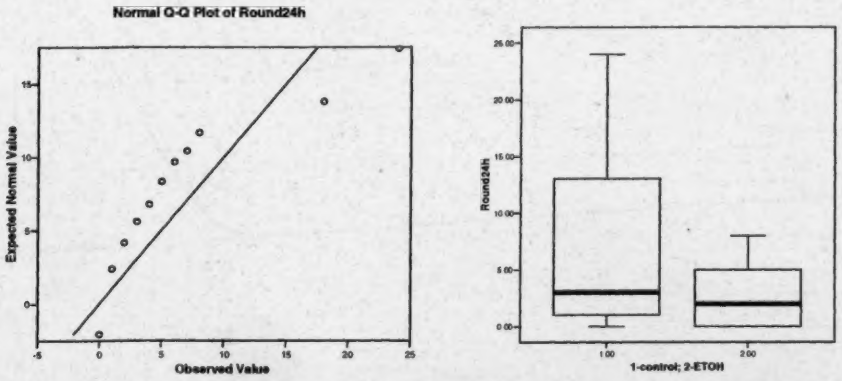


Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

Page



Markedly skewed data in the ETOH group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)



Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

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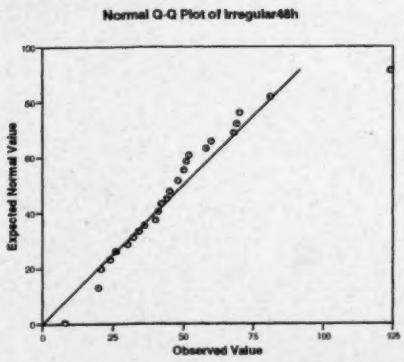
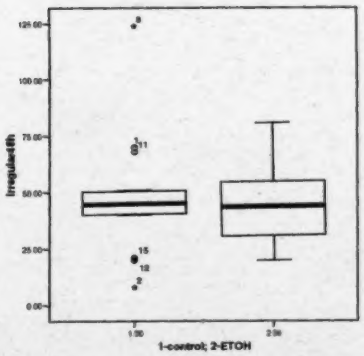
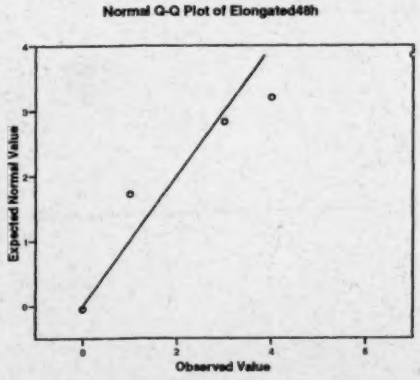
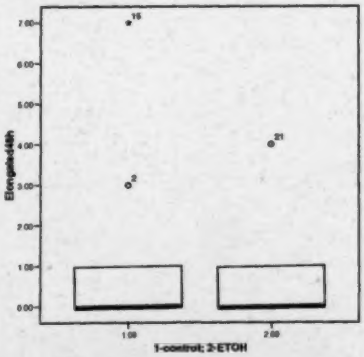
48h interval



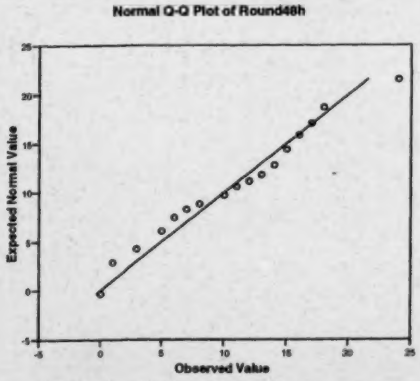


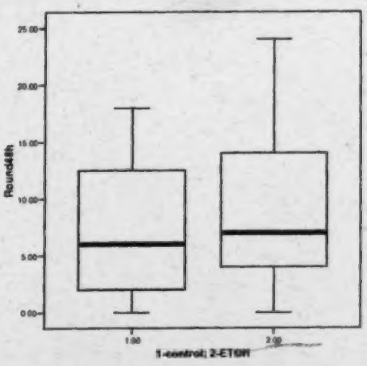
Markedly skewed data in the control group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)





Markedly skewed data in both groups (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)





Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Irregular24h	1.00	15	17.57	263.50
	2.00	15	13.43	201.50
	Total	30		
Elongated24h	1.00	15	17.37	260.50
	2.00	15	13.63	204.50
	Total	30		
Round24h	1.00	15	17.77	266.50
	2.00	15	13.23	198.50
	Total	30		
Irregular48h	1.00	15	15.97	239.50
	2.00	15	15.03	225.50
	Total	30		
Elongated48h	1.00	15	15.27	229.00
	2.00	15	15.73	236.00
	Total	30		
Round48h	1.00	15	14.87	223.00
	2.00	15	16.13	242.00
	Total	30		

Test Statistics[b]

	Irregular24h	Elongated24h	Round24h	Irregular48h	Elongated48h	Round48h
Mann-Whitney U	81.500	84.500	78.500	105.500	109.000	103.000
Wilcoxon W	201.500	204.500	198.500	225.500	229.000	223.000
Z	-1.289	-1.194	-1.432	-.291	-.164	-.396
Asymp. Sig. (2-tailed)	.197	.233	.152	.771	.870	.692
Exact Sig. [2*(1-tailed Sig.)]	.202[a]	.250[a]	.161[a]	.775[a]	.902[a]	.713[a]

a. Not corrected for ties.

b. Grouping Variable: 1-control; 2-ETOH

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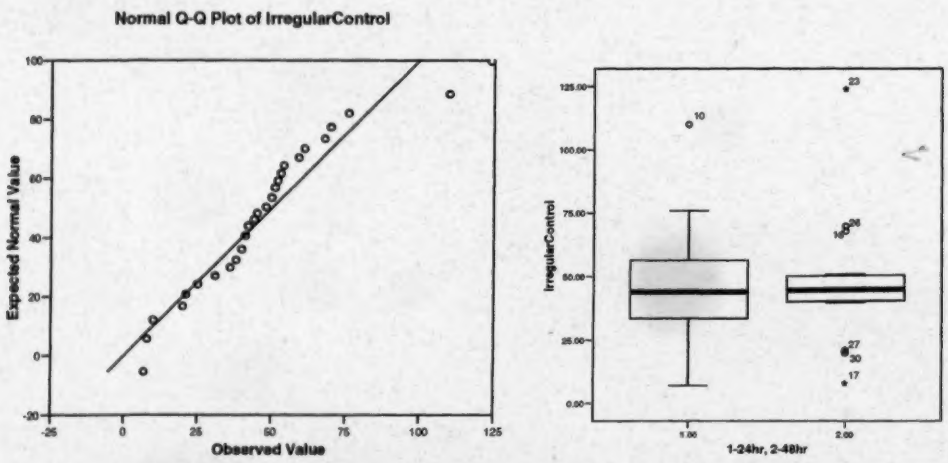
4.2.2 *Comparison of control and alcohol-treated trunk neural crest cells between intervals*

Group Statistics

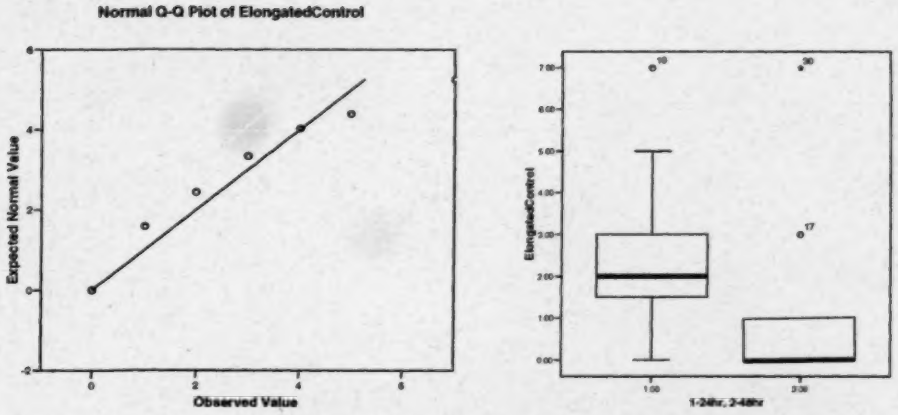
	1-24hr, 2-48hr	N	Mean	Std. Deviation	Std. Error Mean
IrregularControl	1.00	15	46.4000	25.66766	6.62736
	2.00	15	47.9333	26.72203	6.89960
ElongatedControl	1.00	15	2.4000	1.91982	.49570
	2.00	15	.9333	1.86956	.48272
RoundControl	1.00	15	7.4000	8.87855	2.29243
	2.00	15	7.4000	6.40089	1.65270
IrregularETOH	1.00	15	37.0000	30.17568	7.79133
	2.00	15	43.9333	17.58842	4.54131
ElongatedETOH	1.00	15	1.8000	2.45531	.63396
	2.00	15	.6667	1.04654	.27021
RoundETOH	1.00	15	2.6667	2.94392	.76012
	2.00	15	8.6667	7.00680	1.80915

Control group

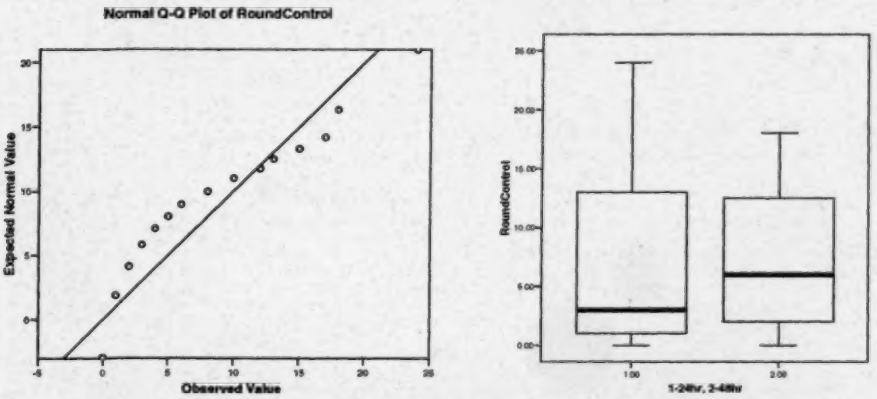


Markedly skewed distribution of the 48h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

Page

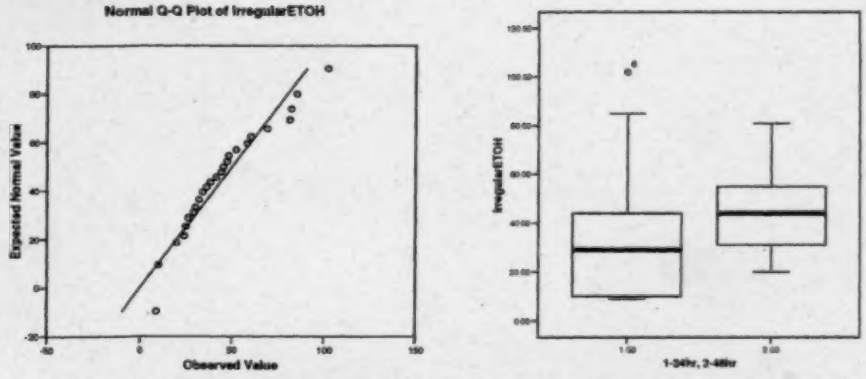


Markedly skewed data in the 48h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

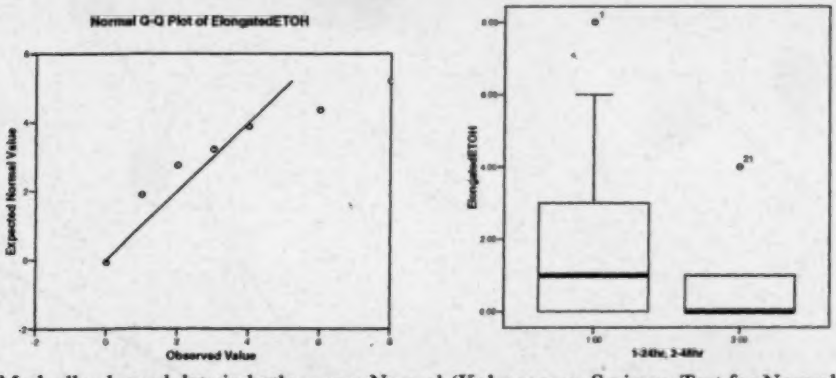


Markedly skewed data in the 24h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)

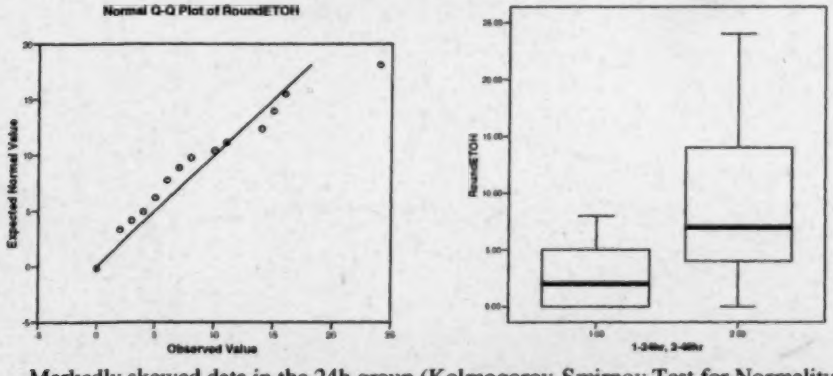
Page
ETOH group



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), equal variance (Levene's Test for Equality of Variance $p>0.05$)



Markedly skewed data in both groups Normal (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)



Markedly skewed data in the 24h group (Kolmogorov-Smirnov Test for Normality, $p<0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-24hr, 2-48hr	N	Mean Rank	Sum of Ranks
IrregularControl	1.00	15	15.43	231.50
	2.00	15	15.57	233.50
	Total	30		
ElongatedControl	1.00	15	19.50	292.50
	2.00	15	11.50	172.50
	Total	30		
RoundControl	1.00	15	15.10	226.50
	2.00	15	15.90	238.50
	Total	30		
IrregularETOH	1.00	15	13.30	199.50
	2.00	15	17.70	265.50
	Total	30		
ElongatedETOH	1.00	15	17.07	256.00
	2.00	15	13.93	209.00
	Total	30		
RoundETOH	1.00	15	11.53	173.00
	2.00	15	19.47	292.00
	Total	30		

Test Statistics[b]

	Irregular Control	Elongated Control	RoundControl	IrregularETOH	Elongated ETOH	RoundETOH
Mann-Whitney U	111.500	52.500	106.500	79.500	89.000	53.000
Wilcoxon W	231.500	172.500	226.500	199.500	209.000	173.000
Z	-.041	-2.588	-.250	-1.371	-1.054	-2.519
Asymp. Sig. (2-tailed)	.967	.010	.803	.170	.292	.012
Exact Sig. [2*(1-tailed Sig.)]	.967[a]	.011[a]	.806[a]	.174[a]	.345[a]	.013[a]

a. Not corrected for ties.

b. Grouping Variable: 1-24hr, 2-48hr

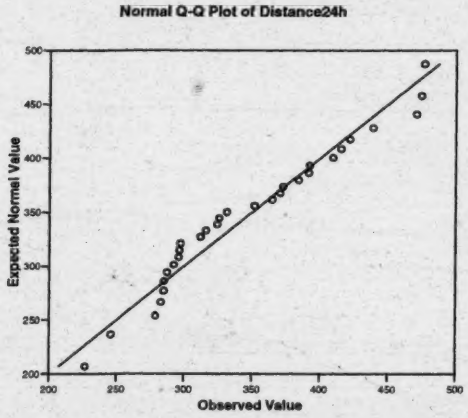
Page

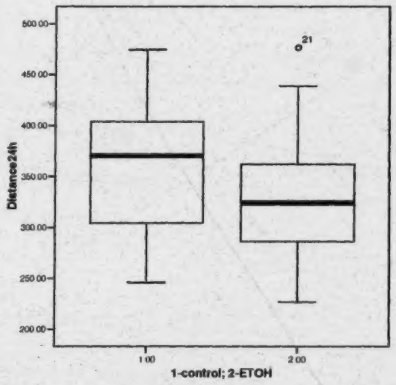
4. 3 Trunk neural crest cells / Collagen / Enriched medium / Distance

4.3.1 Comparison between control and alcohol-treated trunk neural crest cells

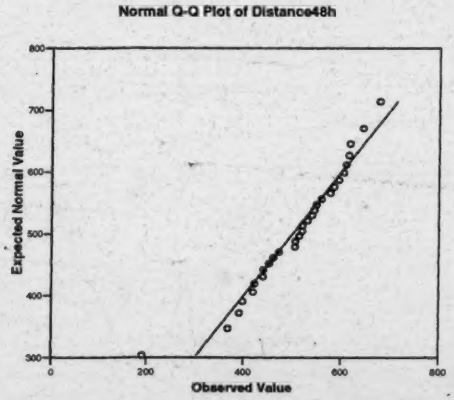
Group Statistics

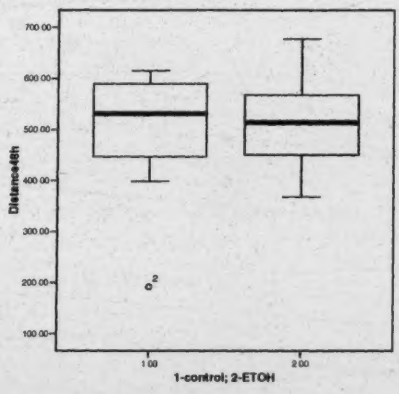
	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Distance24h	1.00	15	362.4632	68.62053	17.71774
	2.00	15	331.9693	67.47235	17.42129
Distance48h	1.00	15	503.7493	111.81791	28.87126
	2.00	15	513.8531	91.38895	23.59653





Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)





Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Distance24h	1.00	15	17.53	263.00
	2.00	15	13.47	202.00
	Total	30		
Distance48h	1.00	15	15.67	235.00
	2.00	15	15.33	230.00
	Total	30		

Test Statistics[b]

	Distance24h	Distance48h
Mann-Whitney U	82.000	110.000
Wilcoxon W	202.000	230.000
Z	-1.265	-.104
Asymp. Sig. (2-tailed)	.206	.917
Exact Sig. [2*(1-tailed Sig.)]	.217[a]	.935[a]

a. Not corrected for ties.

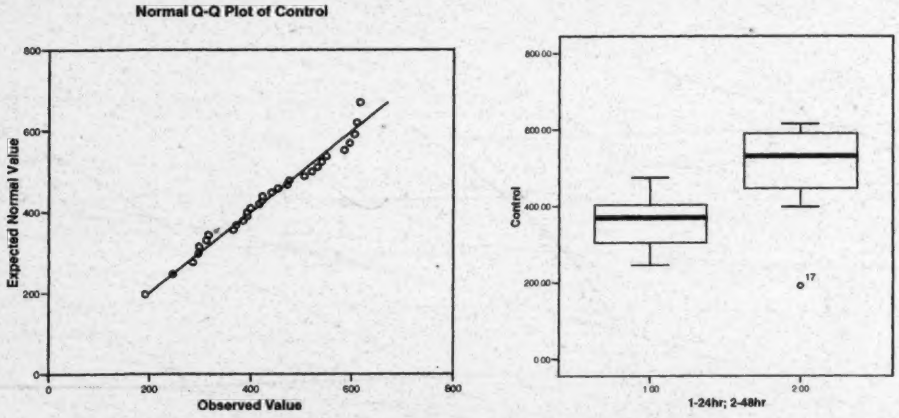
b. Grouping Variable: 1-control; 2-ETOH

Page

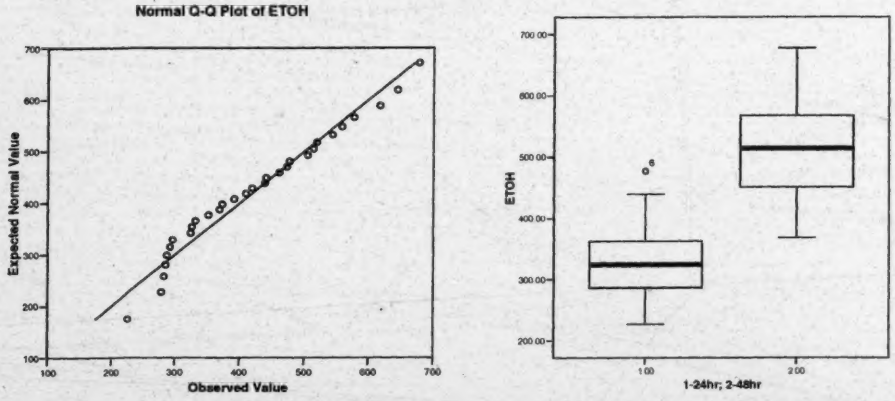
4.3.2 Comparison of control and alcohol-treated trunk neural crest cells between intervals

Group Statistics

	1-24hr; 2-48hr	N	Mean	Std. Deviation	Std. Error Mean
Control	1.00	15	362.4632	68.62053	17.71774
	2.00	15	503.7493	111.81791	28.87126
ETOH	1.00	15	331.9693	67.47235	17.42129
	2.00	15	513.8531	91.38895	23.59653



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-24hr; 2-48hr	N	Mean Rank	Sum of Ranks
Control	1.00	15	9.73	146.00
	2.00	15	21.27	319.00
	Total	30		
ETOH	1.00	15	8.80	132.00
	2.00	15	22.20	333.00
	Total	30		

Test Statistics[b]

	Control	ETOH
Mann-Whitney U	26.000	12.000
Wilcoxon W	146.000	132.000
Z	-3.588	-4.169
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[a]	.000[a]

a. Not corrected for ties.

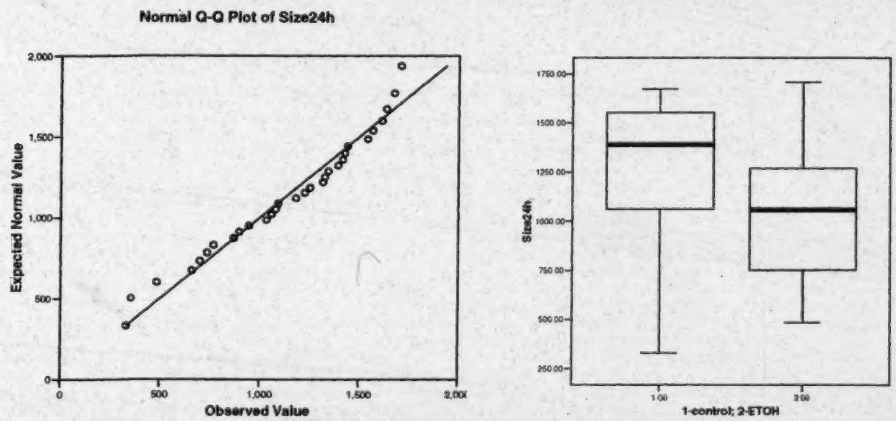
b. Grouping Variable: 1-24hr; 2-48hr

4.4 Trunk neural crest cells / Collagen / Enriched medium / Size

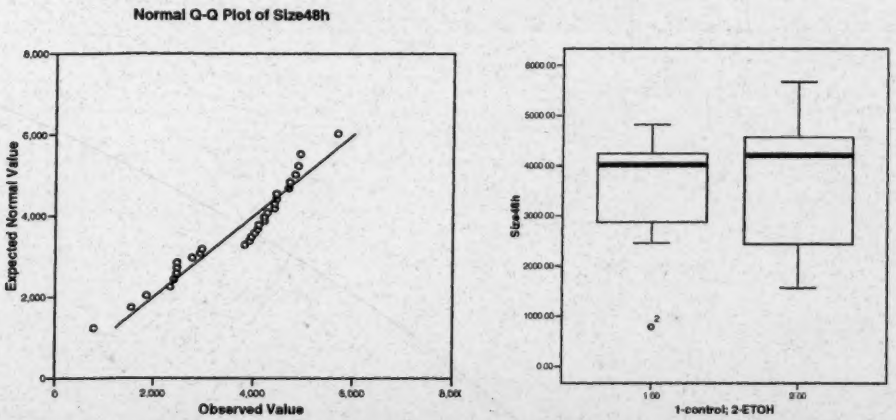
4.4.1 Comparison between control and alcohol-treated trunk neural crest cells

Group Statistics

	1-control; 2-ETOH	N	Mean	Std. Deviation	Std. Error Mean
Size24h	1.00	15	1236.3495	428.87553	110.73519
	2.00	15	1033.5065	338.32447	87.35500
Size48h	1.00	15	3589.8942	1092.24473	282.01638
	2.00	15	3673.5464	1289.29319	332.89407



Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, p>0.05), equal variance (Levene's Test for Equality of Variance p>0.05)



Markedly skewed data in the control group (Kolmogorov-Smirnov Test for Normality, p<0.05), equal variance (Levene's Test for Equality of Variance p>0.05)

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-control; 2-ETOH	N	Mean Rank	Sum of Ranks
Size24h	1.00	15	18.27	274.00
	2.00	15	12.73	191.00
	Total	30		
Size48h	1.00	15	15.00	225.00
	2.00	15	16.00	240.00
	Total	30		

Test Statistics[b]

	Size24h	Size48h
Mann-Whitney U	71.000	105.000
Wilcoxon W	191.000	225.000
Z	-1.721	-.311
Asymp. Sig. (2-tailed)	.085	.756
Exact Sig. [2*(1-tailed Sig.)]	.089[a]	.775[a]

a. Not corrected for ties.

b. Grouping Variable: 1-control; 2-ETOH

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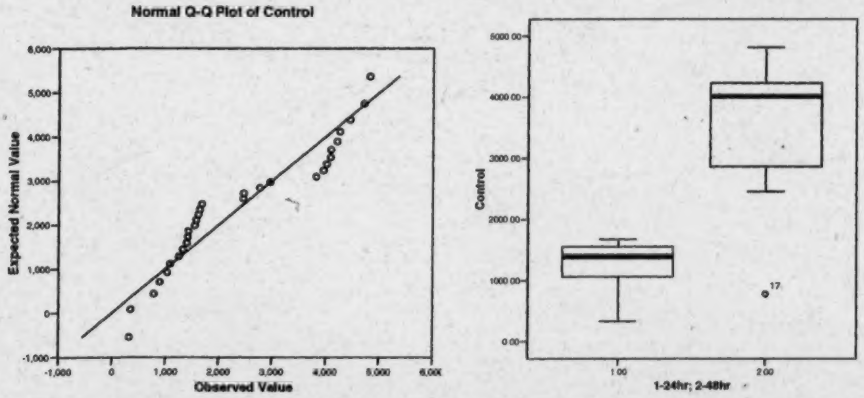
4.4.2 *Comparison of control and alcohol-treated trunk neural crest cells between intervals*

Group Statistics

	1-24hr; 2-48hr	N	Mean	Std. Deviation	Std. Error Mean
Control	1.00	15	1236.3495	428.87553	110.73519
	2.00	15	3589.8942	1092.24473	282.01638
ETOH	1.00	15	1033.5065	338.32447	87.35500
	2.00	15	3673.5464	1289.29319	332.89407



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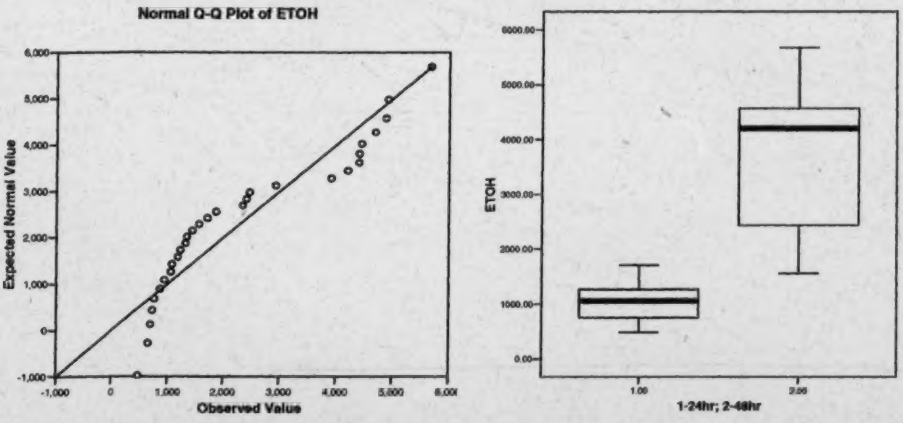

Normal data distribution in both groups (Kolmogorov-Smirnov Test for Normality, $p>0.05$), unequal variance (Levene's Test for Equality of Variance $p<0.05$)

Page

NonParametric Test

Wilcoxon/ Mann-Whitney Test

Ranks

	1-24hr; 2-48hr	N	Mean Rank	Sum of Ranks
Control	1.00	15	8.87	133.00
	2.00	15	22.13	332.00
	Total	30		
ETOH	1.00	15	8.07	121.00
	2.00	15	22.93	344.00
	Total	30		

Test Statistics[b]

	Control	ETOH
Mann-Whitney U	13.000	1.000
Wilcoxon W	133.000	121.000
Z	-4.127	-4.625
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[a]	.000[a]

a. Not corrected for ties.

b. Grouping Variable: 1-24hr; 2-48hr

APPENDIX D

EXPERIMENT PROTOCOL APROVAL

UNIVERSITY OF ALBERTA

ETHICS APPROVAL FOR ANIMAL USE PROTOCOL
FROM THE
HEALTH SCIENCES ANIMAL POLICY & WELFARE COMMITTEE (HSAPWC)

The Health Sciences Animal Policy & Welfare Committee

UNIVERSITY OF ALBERTA

Has reviewed and approved the protocol application entitled:

PROPERTIES OF NEURAL CREST CELLS
Title

B	076/11/05
Category of Invasiveness	Protocol Number

Submitted by:

Dr. Nadine C. Milos
Name of Principal Investigator

And found the proposed protocol involving animals to meet the standards of the Canadian Council on Animal Care (CCAC), and the proposed facilities in which the animals will be housed and used to comply with the CCAC requirements.

Frog	62
Species Strain	Number of Animals Approved
	November 30, 2005
Signature of HSAPWC Chairperson	Expiry Date

Health Sciences Animal Policy and Welfare Committee
218C Campus Tower, 11145-87 Ave, Edmonton, AB T6G 0H1 Canada • Phone: 780-492-2695 • Fax: 780-492-9429